

# ADVANCES IN PROTEIN CHEMISTRY

EDITED BY

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VOLUME III



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# Transamination and the Integrative Functions of the Dicarboxylic Acids in Nitrogen Metabolism

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## INTRODUCTION

Since the discovery of enzymatic transamination in 1937 (32, 33, 34) the development of this subject has been reviewed by Braunstein (23, 24), by Cohen (61, 62) and lately by Herbst (91). In the present article no full account is proposed of earlier findings and hypotheses in this field. It is timely, though, to summarize present knowledge of this metabolic process and to sift well-established facts from erroneous or doubtful conclusions due to imperfect experimental technique or to premature generalization. We also wish to correct certain misinterpretations and misquotations of our data, and to discuss differences of opinion.

To comply with the general purpose of this volume, attention is focused on the role of transamination in amino acid and protein metabolism, with a minimum of purely enzymological information. A rather extended discussion of the metabolic functions of dicarboxylic amino acids and their precursors is included to arrive at a clearer insight into the metabolic interrelations of transamination and its physiological significance, which are still imperfectly understood.

**ABBREVIATIONS.** The following abbreviations are used in the text:

GL — *l*-glutamic acid; AS — *l*-aspartic acid; AL — *l*-alanine; KG —  $\alpha$ -ketoglutaric acid; OA — oxaloacetic acid; PU — pyruvic acid; gl-aph — glutamic aminopherase; as-aph — aspartic aminopherase; co-aph — coenzyme of aspartic aminopherase.

In the symbols denoting the more important transamination reactions, only the initial and final amino acids will be indicated, omitting the participating keto acids, *e.g.*,

$GL \rightleftharpoons AL$ , for: *l*-glutamic acid + pyruvic acid  $\rightleftharpoons$  *l*-alanine +  $\alpha$ -ketoglutaric acid;

$AL \rightleftharpoons AS$ , for: *l*-alanine + oxaloacetic acid  $\rightleftharpoons$  *l*-aspartic acid + pyruvic acid.

#### I. REMARKS ON EXPERIMENTAL METHODS

In view of the lack of specific analytical methods for amino-dicarboxylic acids at that time, disappearance or formation of GL and AS was estimated, in our early studies on transamination, by determinations of  $NH_2-N$  in the aminodicarboxylic acid fraction precipitated according to Foreman. Although admittedly non-specific, this method is sufficiently reliable for roughly quantitative experiments of this type. We still use it for the rapid estimation of relative transamination rates in the absence of interfering substances. The method fails in the presence of certain other amino acids, *e.g.*, tyrosine, cystine, glycine, large amounts of leucine; its accuracy is, of course, insufficient for kinetic measurements or the detection of small changes of GL or AS. Cohen (56) should be thanked for developing a specific micromethod for GL, which enabled him to investigate the rapid reaction  $GL \rightleftharpoons AL$  and delimit the scope of the transamination process more correctly than we had done. Cohen (59, 60) used non-specific differential determinations of  $CO_2$ , liberated with chloramine T from AS (2 moles) and from other amino acids (1 mole), to estimate transformations of AS. In this laboratory, AS has been determined by the method of Fromageot and Heitz (79) with subtraction of the acetaldehyde equivalents of AL, lactic and malic acids. A strictly specific micromethod for AS has now been worked out by Braunstein and Nemchinskaya (38), based on exhaustive methylation of AS (Dakin (66)) and manometric determination of the resulting fumaric acid (113).

AL was formerly determined according to Kendall-Fürth or Fromageot and Heitz (79). Two highly sensitive and convenient micromethods for AL have been made available (Braunstein and Bychkov, 30), combining oxidation of AL to acetaldehyde either with isatin (*resp.* ninhydrin, *cf.*

Virtanen, 163) or according to Fromageot and Heitz, and specific photometry of  $\text{CH}_3 \cdot \text{CHO}$  with *p*-hydroxydiphenyl. The isatin method requires removal of AS, the permanganate procedure eventually involves corrections for lactic acid and for threonine, because deaminated threonine is quantitatively oxidized to  $\text{CH}_3 \cdot \text{CHO}$  by permanganate (30).

KG can be determined quantitatively by the method of Krebs (107) OA—colorimetrically after Straub (153), PU—with carboxylase or by the salicylic aldehyde reaction of Straub (153), cf. Braunstein (22).

Dependable analytic procedures are thus available at present for the major constituents of transamination systems. Caution is necessary, however, in the set-up and interpretation of experiments, to avoid fallacies due to side-reactions. Thus, transformations of preformed amino-N donors or acceptors in animal tissues have been mistaken for slight transamination of *d*-amino acids (75, 76, 26, 52) and of certain other added substrates (35, 36). GL formation from histidine and arginine, included by Cohen (58) in a table of transamination rates, is probably due to other transformations of these amino acids. Doubtful conclusions concerning transamination have been based on indiscriminate use of semiquantitative tests for rapidly metabolized substrates like OA and PU (40, 2, 3, 50, 52). *In every instance, two components, at least, of any transamination system should be determined.* We concur with Herbst (91) as to the desirability of identifying isolated reaction products whenever a new system is being explored.

## II. MECHANISM OF THE TRANSAMINATION REACTION

### 1. *Non-Biological Transamination*

Deamination of amino acids by carbonyl compounds has long been known in organic chemistry. Classical instances are the Strecker reaction of amino acids with alloxan and similar reactions with *o*-quinones, isatin, ninhydrin, methylglyoxal, and the like. In such cases deamination is usually associated with decarboxylation of the amino acid. Condensation of the amino and carbonyl groups to yield Schiff's bases, and tautomeric transformation of these, are generally assumed as intermediary steps.

Formation of Schiff's bases is also postulated in the case of decarboxylation of keto acids by amino compounds (Langenbeck's artificial carboxylases). Recently bacterial amino acid decarboxylases have also been shown to contain an active carbonyl in their prosthetic component—phosphorylated pyridoxal (84).<sup>1</sup>

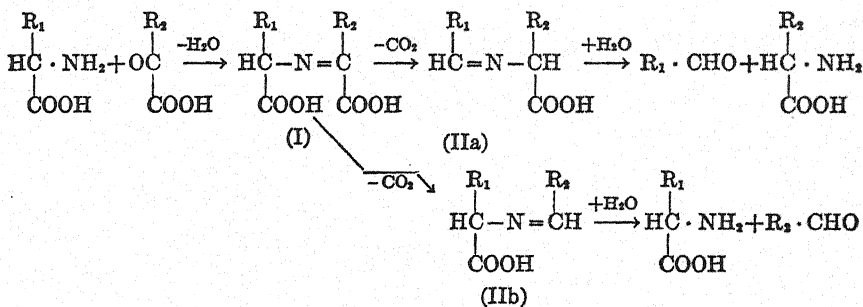
In 1934, Herbst and Engel (92) found that, in boiling aqueous solutions, the  $\text{NH}_2$ - group of  $\alpha$ -amino acids is transferred to  $\alpha$ -keto acids, a new

<sup>1</sup>In a preliminary note, published in January, 1945, F. Schlenk and E. E. Snell (145a) report evidence pointing to the possibility that the system: pyridoxal  $\rightleftharpoons$  pyridoxamine may be involved in enzymatic transamination as an intermediary carrier of amino groups (see pp. 13-14).

amino acid being formed, while the original one is deaminated and decarboxylated to the corresponding aldehyde, *e.g.*:



In many instances, the aldehyde  $\text{R}_2 \cdot \text{CHO}$ , corresponding to the original keto acid, is formed in addition to the aldehyde  $\text{R}_1 \cdot \text{CHO}$ , resulting from breakdown of the initial amino acid. The reaction mechanism is pictured as follows (Herbst, 91):



Scheme 1

Condensation of the substrates to Schiff's base (I) is followed by tautomeric shift of the double bond and simultaneous decarboxylation of the amino acid residue; hydrolysis of the aldmino-compound (IIa) to the end products ensues. Alternatively, the carboxyl adjacent to the double bond in Schiff's base (I) may be split off, and hydrolysis of the aldmino-compound (IIb) will result in recovery of the original amino acid and formation of the aldehyde  $\text{R}_2 \cdot \text{CHO}$ .

The rate of non-biological transamination is highest with aromatic  $\alpha$ -amino acids or cystine, *i.e.*, with substrates inactive in enzymatic transamination (89). On the other hand, the dicarboxylic amino and  $\alpha$ -keto acids react slowly in this model system (88, 91), whereas their participation is obligatory in enzymatic transamination (34, 23).

Non-biological transfer of amino groups has also been effected between  $\alpha$ -amino acids and the carbonyl of  $\alpha$ -ketoacylamino acids (Herbst and Shemin, 94); no well-established instance of reactions of this type in enzymatic systems is known as yet.

Recently, Brewer and Herbst (43) studied alkali-catalyzed transaminations in non-aqueous solution between the esters of amino and  $\alpha$ -keto acids. These reactions are more closely analogous to enzymatic transamination, insofar as the amino acid residue is not decarboxylated and the reaction is strictly reversible.

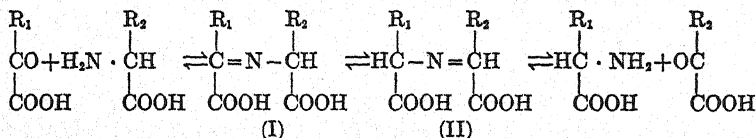
## 2. Enzymatic Transamination

The most extensively studied enzymatic transamination reactions of major biological importance in animal tissues are:

- (1)  $GL + PU \rightleftharpoons AL + KG$  (Braunstein and Kritzmman, 33, 34).
- (2)  $GL + OA \rightleftharpoons AS + KG$  (Szent-Györgyi and Banga, 10; Cohen 57).
- (3)  $AS + PU \rightleftharpoons AL + OA$  (Karyagina, 99; Cohen and Hekhuis, 64).

These reactions have been shown to be reversible equilibrium reactions. Their configurational asymmetry has been demonstrated by the isolation and polarimetric investigation (contrary to the critical allusion of Herbst, 91) of pure *l*-GL (hydrochloride),  $\alpha_D^{20} = +30.9^\circ$ , resp.  $+32.3^\circ$ , in the reactions:  $dL$ -, resp.  $l$ - $AL \rightleftharpoons GL$  (24, 26), and of pure toluenesulfonyl-*l*-AL,  $\alpha_D^{16} = +7.5^\circ$ , in the reaction  $AS \rightleftharpoons AL$  (99).

The reaction scheme of enzymatic transamination assumed by Braunstein and Kritzman (34) is analogous to the mechanism postulated by Herbst for the non-biological reaction (92, 91):



Scheme 2

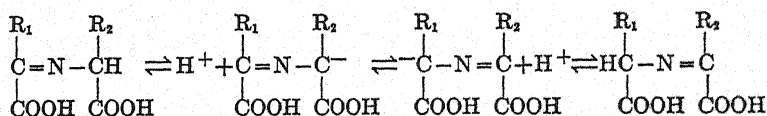
The intramolecular oxidoreduction, or reversible prototropic change in the methylene-azomethine bridge (interconversion of Schiff's bases I and II) is thought to be the enzymatically catalyzed step.

Direct evidence for the spontaneous condensation of amino and keto acids in aqueous media was provided by Knoop and Martius (100) in their synthesis of a diastereoisomer of octopine (cf. Herbst, 91) by catalytic hydrogenation of a solution of arginine and PU. However, arginine is inactive in enzymatic transamination (24). Karrer and associates (96) maintained that octopine is oxidized in minced liver tissue, and suggested that transamination might involve intermediary hydrogenation of Schiff's base I to an  $\alpha\alpha$ -iminodicarboxylic acid (octopine analog) and subsequent dehydrogenation to Schiff's base II. They also assumed that  $\alpha\alpha$ -iminodipropionic acid and its homologs may act as intermediates in oxidative deamination of *l*-amino acids (97), but negative results of later more careful experiments by Karrer and Appenzeller (98) caused them to abandon this hypothesis.

## 3. Transamination Studies with Deuterium

The mechanism of hydrogen transfer in enzymatic transamination has been studied by Konikova, Kritzmman and Teiss (102) in experiments with  $\alpha$ -deuterio-alanine. In the presence of crude gl-aph and of KG the greater part of the deuterium contained in labeled AL passes into the water; the newly formed GL contains practically no excess of D. No significant loss of

D from  $\alpha$ -deuterio-AL was observed in control experiments without enzyme or without KG. This indicates that in transamination the interconversion of the intermediate Schiff's bases I and II is a typical prototropic change, involving labilization and dissociation of the  $\alpha$ -hydrogen, followed by electromeric shift in the methylene-azomethine bridge and by uptake of a proton from water in the  $\alpha$ -position of the newly formed amino acid residue, as in the formulation (91):



Scheme 3

A detailed reinvestigation (Konikova, Dobbert and Braunstein, 39) with  $\alpha$ -deuterio-*dl*-AL and  $\alpha$ -deuterio-*l*-GL and highly purified gl-aph (prepared according to Lenàrd and Straub, 125) corroborates and extends these findings, and shows that the labilization of  $\alpha$ -hydrogen is an independent phenomenon, catalyzed by the transamination enzyme, in certain cases even under conditions excluding any analytically detectable electron shift, *e.g.*, in experiments with gl-aph inactivated by 15 minutes' boiling, or with GL in the absence of  $\alpha$ -ketoacids. The results can be briefly summarized as follows:

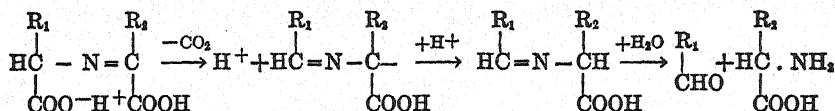
Substrates	With active gl-aph		With boiled gl-aph		Controls without enzyme	
	$\alpha$ -hydrogen exchange	Trans-amination	$\alpha$ -hydrogen exchange	Trans-amination	$\alpha$ -hydrogen exchange	Trans-amination
GL+PU	++++	++++	++++	—	—	—
GL+KG	++++	Not detectable by chemical analysis	++++	—	—	—
GL	++++	—	++++	—	—	—
AL+KG	++++	++++	++++	—	—	—
AL+PU	+++	Not detectable	+++	—	—	—
AL	—	—	—	—	—	—

No explanation is offered at present for the mechanism of  $\alpha$ -hydrogen activation, for the lack of symmetry with respect to the behavior of GL



and of AL, etc. The study of the mechanism and specificity of the  $\alpha$ -hydrogen labilization reaction is being continued. (See Addendum 1, p. 52a.)

In the non-biological model system, the  $\alpha$ -hydrogen of the original amino acid does not enter into reaction, as shown by the isotope experiments of Herbst and Rittenberg (93). When phenylglycine was transaminated with PU in boiling heavy water, the resulting AL took up D in the  $\alpha$ - and  $\beta$ -positions, while benzaldehyde free of excess deuterium was formed. With  $\alpha$ -deuteriophenylglycine and PU in  $H_2O$ , the benzaldehyde retained most of the D present in the labeled phenylglycine, and the AL formed was free of excess isotope. In this case the reaction evidently proceeds through the following steps:



Scheme 4

In this scheme the carboxyl group of the original amino acid serves as the source of the dissociated proton and of the electron involved in the electromeric shift. The uncharged OOC-group is liberated as  $CO_2$  simultaneously with the shift of the double bond.

#### 4. Comparison of Non-Biological and Enzymatic Transamination

The model reaction of Herbst and enzymatic transamination are similar insofar as the transfer of nitrogen in either case is based on intermediary formation and prototropic rearrangement of Schiff's bases.

The principal points of distinction between the two types of transamination are indicated below:

##### Non-biological transamination:

1. Proceeds uncatalyzed at boiling temperature.
2. Most rapid in acid solution; completely inhibited at neutral or alkaline reaction.
3. Irreversible owing to decarboxylation of amino acid.
4. Electromeric change at the expense of electron from ionized carboxyl group of amino acid; uncharged OOC-group detached as  $CO_2$ ;  $\alpha$ -hydrogen of original amino acid retained in resulting aldehyde;  $\alpha$ -position of new amino acid filled by a proton ( $H^+$ -ion) from aqueous medium.

##### Enzymatic transamination:

1. Catalyzed by enzymes; proceeds rapidly at low temperature.
2. Optimal pH 7.5; complete inhibition below pH 3.5 and above pH 9.5.
3. Perfectly reversible; involves no decarboxylation.
4. Electromeric change at the expense of electron from  $\alpha$ -carbon of amino acid, after dissociation of labilized  $\alpha$ -hydrogen as  $H^+$ -ion;  $\alpha$ -position of new amino acid filled by a proton from aqueous medium.

5. No specificity for configuration. Even with optically active original amino acid, the newly formed amino acid is racemic.

6. Monocarboxylic acids react more readily than dicarboxylic ones. Aromatic amino acids and cystine are transaminated most readily.

7. Amino nitrogen can be transferred to  $\alpha$ -ketoacylamino acids with the formation of peptides.

5. Stereochemically asymmetric; original amino acid must belong to *l*-series; newly formed amino acid has the same configuration.

6. One of the initial substrates must be a dibasic acid. Highest velocity attained with two dicarboxylic substrates. Aromatic amino acids, diamino acids, cystine and hydroxyamino acids fail to react.

7. According to the more reliable data, peptides and  $\alpha$ -ketoacylamino acids are not active in enzymatic transamination.

### III. ENZYME SYSTEM OF TRANSAMINATION

#### 1. Terminology

Transamination in biological systems is catalyzed by a special group of desmolases. These enzymes are not identical with *l*- or *d*-amino acid oxidase or with the specific dehydrogenases of GL or AS, although they may have certain components in common with some of these. The writer (24) termed the enzymes of transamination "*aminopherases*" in conformity with their mode of action. There exist at least two different aminopherases, obtained as cell-free preparations and partially purified by M. G. Kritzmann. One of these, *glutamic aminopherase* (gl-aph), catalyzes transaminations involving GL, resp. KG (115). (This preparation contains two individual enzymes of narrower specificity, cf. p. 10 and Addendum p. 52a and 52b.) The other, *aspartic aminopherase* (as-aph), effects transaminations of AS, resp. OA (117).

P. P. Cohen (59), who obtained a preparation of gl-aph by essentially the same method as Kritzmann and contested the existence of as-aph, has advanced the name "*transaminase*" for his preparation. There is no valid reason for this revision of the term proposed by the first investigators and accepted by leading enzymologists (Oppenheimer; Nord and Weidenhagen, 133; D. E. Green, 81) and by all European and many American authors. Cohen's arguments are: euphony, and the avoidance of mixed etymology. The first point is a matter of taste, but the name aminopherase is constructed conformably to the accepted nomenclature of enzymes with analogous action, *e.g.*, phosphopherases, hydrokinases or hydrogen transportases (133) rather than "transphosphorases," "transhydrases." As regards hybrid etymology, the purist is free to obviate this by altering the spelling "aminopherase" to "aminoferase," a word of untainted Latin style, or to the hellenized "amminopherase."

#### 2. Glutamic Aminopherase

Kritzmann (115) obtained clear solutions of gl-aph from extracts of pigeon breast muscle or pig heart by a procedure involving isoelectric precipitation of the enzyme at pH 4.5, denaturation of inactive proteins

at 60° C. and partial purification by salting out with ammonium sulfate and dialysis. The enzyme catalyzes the reactions  $GL \rightleftharpoons AL$  (115) and  $GL \rightleftharpoons AS$  (59). Dry preparations are obtained by rapid desiccation *in vacuo* (preferably, in the frozen state); dehydration with acetone or alcohol destroys the enzyme. Similar, but less active preparations made by Cohen (59) were inactivated by salting out or dialysis, but could be further purified by adsorption on calcium phosphate (61).

The enzyme is not impaired by 20 minutes' heating to 70° C., but it loses 90% of its activity in 20 minutes at 85° C. The optimal pH is 7.5 in either of the reactions  $GL \rightleftharpoons AL$  (115) and  $GL \rightleftharpoons AS$  (60); the temperature optimum is about 40° C. (60).

Lenàrd and Straub (125) purified gl-aph 570-fold by the following fractionation steps:

Extraction of minced pig heart with *M*/10 acetate buffer at pH 3.8, heating to 60° C., neutralization of strained extract to pH 7, centrifugation (A); precipitation of gl-aph by 0.375 saturation with ammonium sulfate, dialysis (B); adsorption on alumina *Cy* at pH 4.6, elution with phosphate at pH 6.8 (C); fractionation with ammonium sulfate between 0.30 and 0.375 saturation, dialysis (D). The results of this treatment are tabulated as follows:

Stage of purification	Enzyme units*	
	Total	Per mg. dry weight
Initial muscle (800 g.)	8000	0.05
A	4450	1.03
B	2150	4.17
C	937	12.6
D	618	28.3

\* One unit of gl-aph is defined as the amount of enzyme converting 1 mg. of PU into AL in 15 minutes under specified conditions.

For reaction  $AL \rightarrow GL$ , Lenàrd and Straub found a pH-optimum at 7.2, while the optimal pH for the reverse reaction was shifted to the alkaline side. Since the velocity of reaction  $AL \rightarrow GL$  at different  $H^+$ -ion concentrations to the acid side of the optimum is proportional to the percentage of negative AL-ions, Lenàrd and Straub conclude that the enzyme reacts only with the anions, not with the bipolar ions or cations of AL.

Since no further increase of activity could be achieved, the authors consider the enzyme as essentially pure. The final preparation shows no absorption bands (125) or fluorescence (39). (Cohen, 61, found transaminating activity to be associated with protein fractions exhibiting green fluorescence of the flavoprotein type.) Lenàrd and Straub's preparation, in contrast to those of Kritzmman and Cohen, fails to effect the reaction  $GL \rightleftharpoons AS$ . This important fact indicates either the presence in the latter preparations of two distinct aminopherases,  $aphGL \rightleftharpoons AL$  and  $aphGL \rightleftharpoons AS$ ,

or the participation, in reaction  $GL \rightleftharpoons AS$ , of additional factors removed by the treatment of Lenàrd and Straub. (See Addendum 2, p. 52a.)

The  $Q_{\text{transamination}}$  values of starting materials and purified gl-aph preparations, computed after the papers of the quoted authors, are shown in Table I.

TABLE I

Authors	$Q_{AL \rightarrow GL}$		$Q_{GL \rightarrow AS}$		$Q_{AS \rightarrow AL}$	
	Pigeon breast muscle	gl-aph	Pigeon breast muscle	gl-aph	Pigeon breast muscle	gl-aph
Kritzmann	33 (34)	500 (115)	....	....	>3 (39)	0
Cohen	39 (57)	200 (60)	450 (61)	1600 (60)	....	0
Lenàrd-Straub	(pig heart)					
	45 (125)	29,000 (125)	....	0	....	0

Gl-aph is inactive in the system  $AS \rightleftharpoons AL$ . The readily extractable specific protein of as-aph is removed in the first step of fractionation (washing of the minced muscle), as explicitly stated by Kritzmann (115). Attempts of Cohen (59) and Lenàrd and Straub to activate gl-aph in reactions  $GL \rightleftharpoons AL$  or  $AS \rightleftharpoons AL$  with thermostable cofactors are based on misinterpretation of Kritzmann's statement (116) concerning the possible presence in gl-aph of a difficultly dissociable component (prosthetic group), analogous to the dialyzable coenzyme of as-aph. This suggestion rests on casual observations indicating that gl-aph activity in certain biological objects (thyroid gland, tissues of thiamine-deficient animals) is sometimes augmented by addition of co-as-aph. Kritzmann (unpublished results) has recently succeeded in obtaining apoenzyme preparations of gl-aph that can be reactivated with boiled tissue extracts; the nature of the prosthetic group is being investigated.

The transfer of amino-N from AS to AL can be effected with gl-aph upon addition of small amounts of KG as intermediary  $NH_2$ -carrier (24, 36). In view of Cohen's failure to reproduce this experiment, the effect has been reinvestigated with determination of AL by a specific method (30) (Table II).

TABLE II

*Reaction  $AS \rightarrow AL$  in the presence of gl-aph and KG (39)*  
1 h. 30 min. at 37° C.

5 ml. gl-aph, phosphate buffer pH 7.5; total volume 8 ml., added substrates:	AL formation $\mu M$	Turnover of KG
A. 200 $\mu M$ GL+200 $\mu M$ PU	81	...
B. 200 $\mu M$ AS+200 $\mu M$ PU	0	...
C. 200 $\mu M$ AS+200 $\mu M$ PU+2 $\mu M$ KG	10.2	5.1
D. 200 $\mu M$ AS+200 $\mu M$ PU+ 5 $\mu M$ KG	17.3	3.4
E. 200 $\mu M$ AS+200 $\mu M$ PU+10 $\mu M$ KG	22.1	2.2
F. 200 $\mu M$ PU+10 $\mu M$ KG (control)	0	...

From animal organs other than muscle (liver, kidney) cell-free extracts of gl-aph but no purified preparations have been obtained; in these objects the enzyme is very fragile (39). An extract from oat seedlings, obtained by Albaum and Cohen (5), had a  $Q_{GL \rightarrow AS}$  value of 905, with a pH optimum at 8.6.

### 3. Aspartic Aminopherase

Press-juice from pig heart is the richest source of this enzyme; it undergoes considerable self-activation upon autolysis at 0° C. or at 37° C. From the autolyzed press-juice Kritzmann (117) prepared active as-aph solutions by coagulation of inert proteins at 60° C.; some purification could be achieved by dialysis, by fractional precipitation with ammonium sulfate or with acetone and CO<sub>2</sub> at 0° C., by adsorption on alumina C $\gamma$ , or on Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>, and elution with phosphate at pH 7.8. All these operations lead to disappearance of as-aph activity, as also does 10–15-fold dilution of the press-juice. Reaction AS  $\rightarrow$  AL is restored when such apo-enzyme solutions are supplemented with boiled tissue extracts or coenzyme concentrates. (See Addendum 3, p. 52b.)

The heat-resistance of as-aph is similar to that of gl-aph. The enzyme is active in the range of pH from 5.5 to 8.5, with a sharp optimum at pH 7.4. Reaction AS  $\rightarrow$  AL is relatively slow, reaching a maximum transformation of 58–60% in two hours. Fully active gl-aph is still present in all apo-as-aph preparations of animal origin. Solutions containing only as-aph could be obtained by extraction of sliced pea seedlings with bicarbonate solution, whereas ground seedlings yielded extracts in which gl-aph was also present. Separation of the plant aminopherases into apo- and coenzyme has not been attempted. They are inactivated at lower temperatures (56° C.) than aph from animal tissues.

The marked reduction in rate of reaction AS  $\rightarrow$  AL (as contrasted to GL  $\rightleftharpoons$  AS or GL  $\rightleftharpoons$  AL) in diluted liver homogenates, noted by Cohen and Hekhuis (63), is readily explained by coenzyme dilution without any assumptions concerning special accessory mechanisms; the very low values of these authors for as-aph activity in other tissues at tenfold dilution are probably attributable to the same cause. The presence of an active aspartic aminopherase (apparently non-dissociated) in pigeon liver is reported by Moulder, Vennesland and Evans (131a).

a. *Coenzyme of Aspartic Aminopherase.* Concentrates of co-as-aph were prepared by Braunstein and Kritzmann (37) from boiled extract of pig heart.

Inactive matter is successively removed with lead acetate, mercuric acetate and silver nitrate in weakly acid solution. The active fraction is then precipitated with silver in alkaline medium, treated with H<sub>2</sub>S and concentrated *in vacuo*; 0.001 ml. of the concentrate is equivalent in activity to 1 ml. of boiled heart extract (Fig. 1).

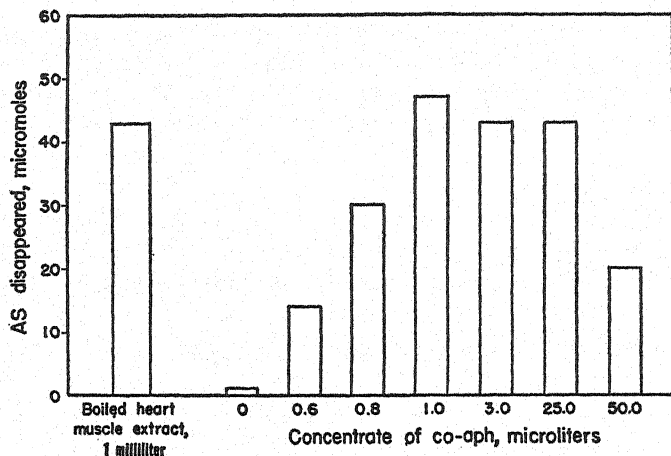
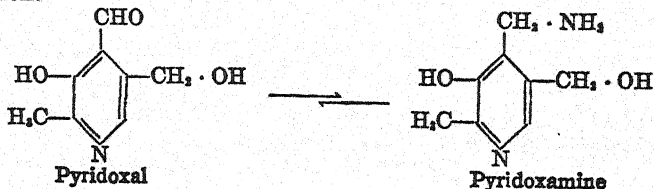


FIG. 1. Activity of aspartic aminopherase (reaction  $AS \rightarrow AL$ ) in relation to concentration of co-aph (Braunstein and Kritzmann, 37).

Co-aph is a basic water-soluble substance, resistant to heat in alkaline solution and to oxidation with  $H_2O_2$  or alkaline permanganate, but fairly unstable in acid solutions ( $N/1$   $HCl$  inactivates in 5–10 min. at  $100^\circ C$ . and in 1 hr. at room temperature). Activity is markedly impaired by treatment with  $NaOI$ ,  $NaOBr$ ,  $K_3Fe(CN)_6$  or  $Na_2SO_3$ . The concentrates are still heavily contaminated with creatine and give a positive orcinol reaction for pentose. Co-aph could not be replaced by ashed concentrate or by any known enzyme activator or muscle extractive. Further investigation of as-aph and its coenzyme had been suspended in view of war-time conditions.

A possible relationship between the prosthetic component of transaminating enzymes and vitamin  $B_6$  is indicated by the recent investigations of Snell (150a, 145a). Snell (150a) demonstrated that "pseudo-pyridoxine" — the fully active  $B_6$  factor for lactic acid bacteria — can be replaced either by pyridoxal (the synthetic aldehyde derivative of pyridoxine) or by pyridoxamine (the corresponding amine). He postulates a reversible interconversion of these compounds and suggests that they might function as an intermediary system of  $NH_2$ -transfer in biological transamination.



Snell (150b) also demonstrated the non-enzymatic transamination  
 $GL + \text{pyridoxal} \rightleftharpoons KG + \text{pyridoxamine}$

in autoclaved aqueous solutions of the components, by the preparative isolation of all reaction products of the direct and reverse reaction, and also by differential microbiological tests for the vitamin B<sub>6</sub> derivatives.

In a preliminary note Schlenk and Snell (145a) reported that the rates of reaction GL→AS are consistently lowered in the tissues of vitamin B<sub>6</sub>-deficient rats (to about 60–70% of the normal values), and can be restored to the normal level by treatment of the animals with pyridoxine. It should be noted, however, that transamination activity is even more markedly inhibited in the tissues of thiamine-deficient pigeons and rats, and rapidly restored by the administration of thiamine (Kritzmann, 118, 119). In 6 of 9 experiments, Schlenk and Snell succeeded in increasing the gl-aph activity of B<sub>6</sub>-deficient tissues *in vitro* to the extent of 10–50% by the addition of pyridoxal (not of pyridoxamine) together with adenosinetriphosphate, the latter being essential. Gl-aph preparations obtained according to Cohen were shown to contain 8.6 to 12.7γ of vitamin B<sub>6</sub> per gram (assay by yeast growth method). The authors infer that some derivative of pyridoxal is involved in transamination either as a coenzyme or in a less direct manner.\*

The coenzyme of bacterial amino acid decarboxylases evidently is also a phosphorylated pyridoxal derivative (84, 9a, 154a). Cohen and Lichtstein (65a) found no decrease of the rate of reaction GL→AS in suspensions of pyridoxine-deficient *Str. faecalis* R., exhibiting markedly lowered tyrosine decarboxylase activity. Dr. E. F. Gale (personal communication), to whom we sent boiled and lyophilized preparations of gl-aph (prepared according to Lenàrd-Straub), found that the codecarboxylase activity of 6 mg. gl-aph (=108 Lenàrd-Straub units) was equivalent to the activity of 0.5–0.6γ of synthetic phosphopyridoxal; growth assays for vitamin B<sub>6</sub> with *Kloeckera brevis* on the same boiled enzyme, performed by Dr. M. Chance, indicate a content of B<sub>6</sub> equivalent to 0.39γ pyridoxine in 6 mg. Braunstein and Kritzmann (37a) assayed the activity of a partially purified preparation of Pb-codecarboxylase from yeast and of synthetic phosphopyridoxal (both preparations kindly supplied by Dr. E. F. Gale), and also of ATP+pyridoxal hydrochloride (courtesy of Dr. E. E. Snell) in the system: aspartic aph—AS—PU. While the codecarboxylase preparation was fairly active in replacing coaminopherase, very slight activation effects were obtained with pyridoxal (100–200γ) and ATP, or with phosphopyridoxal (20–100γ); coaminopherase subjected to extraction with phenol and precipitation as Pb-salt (steps from the purification procedure of Gale and Epps for codecarboxylase) was practically inactive. These data would seem to indicate that coaminopherase is either different from, or more complex than, phosphorylated pyridoxal.\*

\* For further references, see Editors' note, p. 52 and Author's Addendum, p. 52a, b.

## 4. Effect of Chemical Agents on Enzymatic Transamination

a. *Enzyme Inhibitors.* Chemical inhibition has been studied almost exclusively with gl-aph. Casual observations with as-aph indicate that both enzymes are similarly affected by many reagents. Gl-aph is remarkably resistant to the action of most enzyme poisons and other chemical agents. The influence of various compounds, investigated by Braunstein and Kritzmann (33) and Vyshepan (164) with reaction GL→AL and by Cohen with reactions AL→GL (57) or GL→AS (59), can be summarized as follows:

Practically *no inhibition* is caused by: *narcotics* and *dehydrogenase inhibitors*: saturated aqueous solutions of chloroform, toluene, capryl alcohol, 10–15% ethanol or acetone, urethane, 0.02 *M* sodium arsenate, arsenite, selenite, fluoride, iodo- and bromoacetate; *heavy metal complex forming reagents*: 0.01 *M* pyrophosphate, 0.02 *M* hydrogen sulfide or cysteine, 0.05 *M* glutathione; by the following *reducing and oxidizing agents*: 0.01 *M* ascorbic acid, ferrocyanide, ferrous sulfate, methylene blue, ferric chloride; *carbonyl reagents* (incubated with enzyme before substrate addition): 0.002 *M* semicarbazide, hydroxylamine or phenylhydrazine; by the *anions and cations*: Cl<sup>-</sup>, Br<sup>-</sup>, J<sup>-</sup>, NO<sub>3</sub><sup>-</sup>, HCO<sub>3</sub><sup>-</sup>, CH<sub>3</sub>·COO<sup>-</sup>, SO<sub>4</sub><sup>2-</sup>, HPO<sub>4</sub><sup>2-</sup>; Na<sup>+</sup>, K<sup>+</sup>, NH<sub>4</sub><sup>+</sup>, Mg<sup>2+</sup> at 0.1 *M* concentration, 0.01 *M* manganese sulfate, 0.04 *M* aluminum sulfate, 0.001 *M* lead acetate.

Positive inhibition effects are listed in Table III.

TABLE III

Chemical Agent	Molarity	Per Cent Inhibition	Chemical Agent	Molarity	Per Cent Inhibition
Ferricyanide	0.002–0.01	10–15	CaCl <sub>2</sub>	0.1	85
Malonate	0.1	10 (AS+GL– 26%) (57)	CaCl <sub>2</sub>	0.04	50
Malonate	0.01	6	CaCl <sub>2</sub>	0.02	25
Cyanide	0.05	— (164) 79 (57)	BaCl <sub>2</sub>	0.2	83
Cyanide	0.01	56 (164) 50 (57)	SrCl <sub>2</sub>	0.04	62
Cyanide	0.001	3 (164) 30 (57)	ZnSO <sub>4</sub>	0.001	36
Cyanide	0.0001	— (164) 12 (57)	CuSO <sub>4</sub>	0.001	85
<i>p</i> -Benzoquinone	0.001	100 (164)	Hg(CH <sub>3</sub> COO) <sub>2</sub>	0.001	89
<i>p</i> -Benzoquinone	0.0002	28	Hg(CH <sub>3</sub> COO) <sub>2</sub>	0.0001	10
Quinhydrone	0.001	71	HgNO <sub>3</sub>	0.01	100
Hydroquinone	0.001	44	HgNO <sub>3</sub>	0.001	70
			HgNO <sub>3</sub>	0.0001	16
			AgNO <sub>3</sub>	0.01	100
			AgNO <sub>3</sub>	0.001	70
			AgNO <sub>3</sub>	0.0001	17

Only the marked inhibitory effect of quinone, hydroquinone and quinhydrone is worthy of mention, along with the moderate inhibition by Ca<sup>2+</sup>,



Ba<sup>++</sup> and Sr<sup>++</sup> and with the strong non-specific toxicity of heavy metals (Cu<sup>++</sup>, Zn<sup>++</sup>, Hg<sup>++</sup>, Hg<sup>+</sup>, Ag<sup>+</sup>). Some other *p*- and *o*-quinones, diphenols and amidophenols are also inhibitory (Kritzmann and Vyshepan, unpubl.).

b. *Role of Active SH-Groups in Glutamic Aminopherase (gl-aph)*. Barron and Singer(13) include gl-aph in an extensive list of enzymes, the activity of which depends on the presence of free SH-groups in the enzyme molecule. This claim is based on inhibition by SH-blocking reagents and reactivation with glutathione: gl-aph is inhibited by 0.001 *M* chloromercuribenzoate to 49%, by 0.0001 *M* aryl-arsines and arsine oxides to 80%; activity is completely restored by 0.01 *M* glutathione.

c. *Competitive Inhibition by Dicarboxylic Acids*. The writer (24) observed, with low concentrations of GL, or of KG, a competitive inhibition of gl-aph by saturated aliphatic acids, taken in excess. Using high substrate concentrations and equivalent amounts of the dibasic inhibitory acids, Cohen (57, 59) failed to observe this effect.

### 5. Kinetics: Enzyme-Substrate Affinity

In muscle tissue or gl-aph preparations reaction  $GL + PU \rightleftharpoons AL + KG$  proceeds with equal velocity from either side and reaches equilibrium at 50% transformation (equilibrium constant=1) in less than 10 minutes with pigeon breast muscle (34), in 60 minutes with gl-aph (115). With Cohen's enzyme preparation (60) equilibrium was not attained in 2 hours, but the ratio of initial reaction velocity constants (monomolecular)

$$\frac{K_{GL \rightarrow AL}}{K_{AL \rightarrow GL}} = \text{equilibrium constant, was about 1.}$$

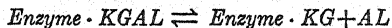
In this reaction, Lenàrd and Straub found 45% PU and 55% AL at equilibrium; using the bimolecular equation, they calculate an equilibrium constant of  $\frac{55^2}{45^2} = 1.43$ .

They state, however, that the observed reaction velocities do not fit in with the curves of either a simple or a reversible bimolecular reaction, and criticize Cohen's statement that transamination velocities can be described by bimolecular velocity constants.

The equilibrium constant of reaction  $GL + OA \rightleftharpoons AS + KG$  is about 3.5 with gl-aph preparations (60). Equilibrium is attained in 30 minutes; at this point the system contains about 75% AS and 25% GL. In muscle tissue an apparent equilibrium is very rapidly established at equal concentrations of AS and GL; Cohen considers this difference as due to the rapid removal of OA in minced muscle by side reactions.

Plotting the rates of reaction  $GL \rightarrow AS$  against substrate concentration (with equivalent amounts of GL and OA), Cohen found for gl-aph preparations a Michaelis constant, *K*, of 0.0138 *M*. In reaction  $AL \rightarrow GL$  Lenàrd and Straub measured the affinity of gl-aph for AL in the presence of an excess of KG, and for KG with AL in

excess. The calculated dissociation constants are 0.015 for the *Enzyme* · *AL* complex, and 0.0017 for *Enzyme* · *KG*. The dissociation constants of *Enzyme* · *GL* and *Enzyme* · *PU* were not determined. Lenàrd and Straub assume that *AL* and *KG* are bound to the enzyme at different points, and that dissociation of the complex with one substrate is not affected by the concentration of the other. In the writer's opinion, only the dibasic reaction partners ("primary" substrates) have direct affinity to their respective aminophosphatases (24), whereas the monocarboxylic partners ("secondary" substrates) combine with the *Enzyme* · *Dicarboxylate* complex to form Schiff's bases.\* From this standpoint only the *Enzyme* · *KG* constant of Lenàrd and Straub is an expression of enzyme-substrate affinity, while the constant for *AL* is the dissociation constant of the Schiff's base:



The kinetics of as-aph action have not yet been studied quantitatively. In homogenized liver, reaction  $\text{AS} \rightarrow \text{AL}$  is 2.19 times faster than the reverse reaction (Cohen and Hekhuis, 64).

\* Our new experiments on the mechanism of  $\alpha$ -hydrogen activation, cf. pp. 6-8, will possibly necessitate an alteration of this conception.

#### 6. Scope of Enzymatic Transamination

In addition to the three most active transamination reactions 1, 2, and 3 (page 6) the following relatively slow reactions occur in minced pigeon muscle: (4)  $\text{GL} \rightleftharpoons l\text{-Aminobutyric acid}$ , (5)  $\text{GL} \rightleftharpoons l\text{-Valine}$ ; (6)  $\text{GL} \rightleftharpoons l\text{-Leucine}$ ; (7)  $\text{GL} \rightleftharpoons l\text{-Isoleucine}$ .

It was at first erroneously assumed by Braunstein and Kritzmann (35) that a number of other monocarboxylic  $\alpha$ -amino- and  $\alpha$ -ketoacids can take part in transamination in muscle. The source of this error, based on unrecognized interactions of *GL*, resp. *KG*, with preformed acceptors and donors of amino nitrogen, has been pointed out by Cohen (57). In our later experiments only the above-mentioned simple homologs of *AL* (resp. *PU*) have been found to be active substrates.

Beside these, the next lower and higher homologs of *OA* and *KG* can act as acceptors of amino groups, and dibasic sulfonic acid analogs of *GL* and *AS* — as donors, in the following reactions:

- |  |      |
|--|------|
| (8) $\text{AL} + \alpha\text{-Ketoadipic acid} \rightarrow \alpha\text{-Aminoadipic acid} + \text{PU}$             | (24) |
| (9) $\text{AL} + \text{Mesoxalic acid} \rightarrow \text{Aminomalonic acid} + \text{PU}$                           | (24) |
| (10) $\text{GL} + \text{Mesoxalic acid} \rightarrow \text{Aminomalonic acid} + \text{KG}$                          | (57) |
| (11) $l\text{-Cysteic acid} + \text{PU} \rightarrow \text{AL} + (\text{Sulfopyruvic acid})$                        | (48) |
| (12) $l\text{-Cysteic acid} + \text{OA} \rightarrow \text{AS} + (\text{Sulfopyruvic acid})$                        | (59) |
| (13) $l\text{-Cysteic acid} + \text{KG} \rightarrow \text{GL} + (\text{Sulfopyruvic acid})$                        | (59) |
| (14) $l\text{-Homocysteic acid} + \text{PU} \rightarrow \text{AL} + (\text{Sulfo-}\alpha\text{-ketobutyric acid})$ | (24) |

Reactions (1), (2), (4) — (10), (12) and (13) are catalyzed by *gl-aph*, reactions (1), (3), and probably (12) — by *as-aph*, whereas reactions (11) and (14) do not take place in enzyme preparations and have been observed only in minced muscle tissue. With pigeon breast muscle Bychkov (48) observed reaction (15)  $\text{Phosphoserine} + \text{PU} \rightarrow \text{AL} + (\text{Phospho-oxypyruvic acid})$ .

acid); Cohen (59) working with a preparation of gl-ph found no indication of this reaction or of transamination between phosphoserine and OA or KG. Since the phosphoserine preparation of Bychkov, obtained from casein, could be contaminated with free or peptide-bound GL, final judgment as to reaction (15) must be postponed until reinvestigation with synthetic phosphoserine.

The specific products of reactions (4) to (15) have not been isolated or exactly characterized.

$\alpha$ -Amino acids of the *d*-series,  $\beta$ - and  $\gamma$ -amino and  $\alpha$ -keto acids, amines, aldehydes and ketones, as well as the peptides thus far tested, are inactive as substrates of transamination (35, 57). It is important that neither glutathione (59) nor asparagine and glutamine (24, 162) can enter into transamination reactions unless previously split by hydrolytic enzymes.

#### IV. DISTRIBUTION AND RATES OF TRANSAMINATION IN BIOLOGICAL SYSTEMS

##### 1. *Animal Tissues*

*a. Relative Rates of Transamination Reactions.* In all biological objects reaction (1)  $GL \rightleftharpoons AS$  is by far the most rapid transamination reaction; the rate of reaction (2)  $GL \rightleftharpoons AL$  is usually 5–10 times slower.\* Reaction (3)  $AS \rightleftharpoons AL$  is the least active, as a rule. The  $Q_{GL \rightarrow AS}$  values are extraordinarily high, reaching 450 in the case of pigeon breast muscle and heart. They exceed the  $Q$  values for succinoxidase and, mostly, for cytochrome oxidase in the same tissues (61). In certain organs, in many tumors and in *E. coli* only reaction (1) proceeds with measurable velocity.

The writer agrees with Cohen's opinion that the extremely high  $Q_{GL \rightarrow AS}$  values indicate an important special function of this reaction in relation to similarly rapid intermediary phases of cellular respiration, involving transformations of dicarboxylic acids. Yet, the velocities of reactions (2) and (3) are also high in some organs, attaining  $Q$  values of 30–45, and 7–10 respectively. Even the rates of slower transamination reactions involving other amino or keto acids are higher (especially in liver or kidney slices) than those of other metabolic transformations of amino acids in the same organs. Insofar as intermediary nitrogen metabolism is concerned,  $Q_{transamination}$  values of the order of 1 to 3 can by no means be considered as insignificant, contrary to the repeated assertion of Cohen.

Most investigations on transamination were not intended to establish maximal or physiological rates of reaction. In fact, only Cohen's experiments had this purpose. It is entirely misleading to arrange side by side, as in the table reproduced by Herbst [(97), p. 89] from Cohen (62), values obtained by different authors under widely differing conditions, *e.g.*, with diluted tissue suspensions and ill-defined extracts, or in prolonged experiments with systems approximating equilibrium.

\*See *Addendum 2* p. 52a.

*b. Distribution of Aminopherases.* Comparable values from Cohen's experiments, representing the activity of different transamination reactions in various animal tissues, are given in Tables IV, V and VI.

TABLE IV

*Transamination between Different Amino Acids and KG  
in Muscle, Liver and Kidney Tissue  
(Adapted from Cohen, 57, 58a)*

Amino-N Donator	Per Cent KG Transformed into GL in 40-60 Mins. at 38° C.		
	Pigeon Breast Muscle	Rabbit Liver	Pig Kidney Cortex
<i>l</i> -Aspartic acid	49	46	55
<i>l</i> -Alanine	40	42	35
<i>dl</i> -Aminobutyric acid	10	—	—
<i>l</i> -Valine	9	12	15
<i>l</i> -Isoleucine	2	4	18
<i>l</i> -Leucine	3	8	10
<i>l</i> -Cysteine	4	0	3
<i>dl</i> -Methionine	1	0	15
<i>l</i> -Phenylalanine	6	8	8
<i>l</i> -Tyrosine	5	—	—
<i>l</i> -Histidine	4	5.6	0
<i>l</i> -Arginine	0	8	19
Glycine, Serine, Tryptophan, Kynurenine (31), Ornithine, Citrulline, Lysine, Threonine (39), Proline, Norvaline, Nor- leucine, and all <i>d</i> -amino acids	0	0	0

TABLE V

*Rates of Reaction AL→GL (mg. GL formed per g. dry tissue) in Different Animal Tissues  
(From Cohen, 58)*

Breast muscle, pigeon	400	Kidney medulla, sheep	16.4
Kidney cortex, guinea pig	72	Amnion, rat	18.8
Testis, rat	60	Pancreas, rat	9.0
Gizzard, pigeon	66	Spleen, guinea pig	4.0
Liver, rat	45	Adrenal cortex, guinea pig	6.6
Brain, guinea pig	26	Uterus, gravid, rat	3.5
Duodenum, rat	24	Adrenal medulla, guinea pig	2.8
Retina, sheep	19	Spleen, rat	2.0
Lung, rat	19		

TABLE VI  
*Q*Transamination in Different Tissues of the Rat (64)

Tissue	$Q_{GL \rightarrow AS}$	$Q_{GL \rightarrow AL}$	$Q_{AS \rightarrow AL}$
Heart muscle	425	7	7
Skeletal muscle	316	13	1
Brain	260	2	8
Liver	245	46	10
Kidney	245	3	3
Testis	150		
Lung	51		
Spleen	16		

It will be seen that transamination activity is very unevenly distributed in different organs. Large generic differences also exist between similar organs in different animals. Reaction  $GL \rightarrow AL$  is absent or negligible in erythrocytes, skin, smooth muscle, pancreas, salivary glands, lymph nodes, placenta, spleen and some other tissues (39); reaction  $GL \rightarrow AS$  is fairly rapid in certain of these (64). There is no definite correlation between aph activity and the functional characteristics or rate of growth of the organs. It is conspicuous, however, that tissues with high transamination rates — namely, muscle, heart, liver, kidney, intestinal mucosa — are those exhibiting a large labile “main respiration” (Battelli-Stern), associated with utilization of the system  $OA \rightleftharpoons \text{Malic acid}$  in respiratory hydrogen transport, *cf.* Breusch (41).

Little is known on the topography of aminopherases in histological elements and on their intracellular localization. Aph activity is higher in brain cortex than in white matter; the cortical layers of kidney and adrenals are more active than the medullar parts of these organs. In kidney, gl-aph appears to be localized in the tubular epithelium (Vyshepan, 165). Unpublished data of Claude (quoted from Schmitt, 146) indicate that aph activity in glandular cells is associated with secretory granules rather than with microsomes.

*c. Transamination in Tumors and Embryonic Tissues.* In malignant tissues reaction  $GL \rightarrow AL$  is either absent or very weak (75, 26); reaction  $GL \rightarrow AS$  usually persists, but its activity is much lower than in normal tissues (75, 63). The configurational specificity of transamination in tumors has been investigated by different authors (76, 26, 63) in view of Kögl's claim that  $GL$  is racemized in the proteins of malignant tissue. In tumors gl-aph is as strictly specific for *l*-amino acids as in normal tissues. Indications of a slight transamination of *d*-amino acids were based on experimental error (see pp. 3–4).

According to Cohen (61) a progressive decline of aph activity attends the development of malignancy of liver tissue in rats fed dimethylaminoazobenzene. He also found that the rates of transamination are low in embryonic tissues of the cat, and concluded that rapid growth is associated with low aph activity, *cf.* p. 31-32.

In the writer's laboratory, Shmerling (149) studied the time of appearance and relative activity of reaction  $GL \rightarrow AL$  in the different organs of the developing rabbit embryo; her findings are summarized in Table VII.

TABLE VII  
*Reaction  $GL \rightarrow AL$  in the Organs of Developing Rabbit Embryos*  
(Shmerling, 149)

Age of Embryos <i>Days</i>	Per Cent Transamination (Average)				
	Liver	Brain	Skeletal Muscle	Heart	Kidney
10-11*	(0)	—	—	—	—
12-13*	(7.0)	—	—	—	—
14-16	11.6	0	0	0	—
17-20	13.6	12.6	0	0	—
21-22	18.5	10.1	0	0	—
23-27	16.8	14.1	12.8	0	0
28	} 22.5	} 13.8	} 21.7	14.3	0
29				18.8	8.3
1-5 after birth	14.4	17.0	24.0	—	22.4

\* Homogenate of whole embryos.

It is seen that gl-aph appears in the embryonic organs at different stages of development: first in the liver, probably as early as hepatic cells are formed, later in brain and muscle, still later in heart, and latest in kidney, where transamination arises about the time of birth.

In certain organs the onset of transamination is definitely coincident with critical stages of functional differentiation, *e.g.*, in muscle — with the time of appearance and accumulation of phosphocreatine and establishment of rapid contraction (Koshtoyants and Ryabinovskaya, 103), in heart — with acquisition of definitive heart rate, in kidney — with the beginning of active excretory function. In the early period of postnatal life transamination values gradually increase, and only at the age of one month do they approach the levels characteristic of adult rabbits.

*d. Effect of Pathological Conditions on Transamination.* Aph activity is remarkably constant in animal tissues under altered trophic and functional conditions, and exhibits a striking resistance to the action of factors inducing pathologic change. The influence of physiological condition and pathogenic agents on the reaction  $GL \rightarrow AL$  in minced tissues has been studied by Vyshepan (165) with the following results:

Gl-aph activity is equal in white and red rabbit muscles; it is not affected by prolonged tetanic stimulation, by denervation or desympathization of the muscle, even by rigor mortis. Normal values were found in muscles of moribund rabbits with transplanted Browne-Pearce tumor, with generalized tuberculosis, or in starvation. Irregular decreases were noted in rabbits injected with tetanus toxin. Gl-aph activity of muscle is undiminished in profound muscle degeneration induced by vitamin E deficiency ("alimentary muscular dystrophy"). In liver, transamination rates per unit of tissue weight are undiminished in severe poisoning with yellow phosphorus (rabbits, acute hepatic degeneration) and with carbon tetrachloride (rats, chronic fatty degeneration and cirrhosis). Allowance being made for the large percentage of necrotized tissue and pathologic deposits in the dystrophic muscles and fatty livers, it follows that gl-aph activity in the persisting remnants of active protoplasm must be considerably augmented. In kidney, transamination is practically abolished in necrotic tubulonephritis induced by uranyl nitrate.

According to S. Kaplansky (95a), the reaction  $GL \rightarrow AL$  is markedly depressed (in parallel with lowered activity of other enzymes) in the liver, kidney and muscles of hypoproteinemic rats, maintained on a protein-deficient diet. After transition to a high-protein diet restitution is very slow.

One instance is known of more or less specific pathological disturbance of transamination, namely, the marked decrease in gl-aph and as-aph activity in all tissues of severely thiamine-deficient pigeons and rats, described by Kritzmann (118, 119); the rates of *L*-amino acid deamination and of "reductive amination" of PU are similarly lowered, while the activity of *D*-amino acid oxidase and of glutamic dehydrogenase is not impaired. Absence of these effects in starved controls and the rapid restoration of aph activity upon administration of thiamine rule out a non-specific effect of lowered protein intake.

Barron and associates (12) found no activation of transamination on addition of thiamine *in vitro* to minced tissues from thiamine-deficient animals, but the experimental conditions were such as to preclude the synthesis of diphosphothiamine. Cohen (61) mentions unpublished results confirming the data of Kritzmann. The decrease of aminopherase activity in pyridoxine-deficient rats has been mentioned on page 14.

## 2. Plants

Information on transamination in plants is very incomplete and chiefly of a qualitative nature. Apart from observations by Kritzmann (114, 117) on the transformations  $GL \rightarrow AL$  and  $AS \rightarrow AL$  in pea, lupine and pumpkin seedlings, the presence of transamination activity in plants, especially in *Leguminosae*, has been demonstrated by Euler and associates (1) and by Virtanen and Laine (160, 162). Cedrangolo and Carandante (53) report that transamination reactions are 1.5–2.5 times more active in dialyzed extracts from seeds and sprouts of *Graminaceae* than of *Leguminosae*, but the reported reaction rates were very low and the analytical methods are

not reliable. Cohen (61) failed to detect transamination in *Chlorella*. In a study demonstrating that the amides of GL and AS are not transaminated until after enzymatic hydrolysis of the amide group, Virtanen and Laine (162) found the following values for reactions  $GL \rightarrow AL$  and  $AS \rightarrow AL$  in crushed pea plants (in 4 hrs. at 40° C.):

	GL	Glutamine	AS	Asparagine
Transamination	31.1%	6.9%	16.7%	1.5%
Deamidation	—	22.6%	—	4.5%

In the developing oat embryo Albaum and Cohen (5) found a rapid increase of gl-aph activity (beginning with the second day of development) parallel with a rise in soluble nitrogen and an acceleration of the rates of protein synthesis. In 4 days  $Q_{GL \rightarrow AS}$ , calculated on the basis of mg. protein, is increased 4-fold and reaches values of the order of 900. The authors infer that there is a direct correlation between transaminating activity and protein synthesis.

Practically nothing is known of the topography of aminopherases in the organs of plants, of their distribution in different genera, and especially of the limits of substrate specificity. A large body of evidence indicates the key position of dicarboxylic acids and their amides in the nitrogen metabolism of the plant. The view that AS and GL are the primary amino acids supplying the nitrogen for the synthesis of other protein constituents (34, 160) is accepted by competent plant physiologists (*cf.* Chibnall, 55). Investigation of the activity of monocarboxylic substrates other than AL, resp. PU, in transamination in plants is, therefore, a problem of urgent importance, especially in view of the narrow scope of transamination reactions thus far demonstrated in animals.

### 3. Microorganisms

Transamination in microorganisms is still less satisfactorily explored than in plants. Reaction  $GL \rightarrow AL$  could not be detected in fresh or plasmolyzed yeast, in the writer's laboratory. In papers from Euler's institute scantily documented data are found on transamination in maceration juice from yeast and in *E. coli* (2, 3). Cohen (61) states that *E. coli* and Lebedew juice from brewer's yeast are active in the reaction  $AS \rightarrow GL$ , but not in  $AL \rightarrow GL$ , while baker's yeast is inactive in both systems. Dicsfalussy (67) failed to detect the reactions mentioned in *E. coli*, *Staphylococcus* or *B. mesentericus*, and Konikova (39) found no transamination of GL or AS with PU in heavy suspensions of *B. brevis* (Dubos' strain BG).

Aubel (oral communication) observed reaction  $AS \rightarrow AL$  anaerobically



with suspensions of *Clostridium sporogenes*; in the presence of oxygen the AL thus formed undergoes oxidative deamination.

Quite recently, Lichstein and Cohen (128a) reinvestigated the transamination activity of various bacteria and showed that the relatively low values of  $Q_{GL \rightarrow AS}$  previously obtained in Cohen's experiments with bacteria, as well as the negative results of Dicsfalussy, were due chiefly to the rapid destruction of substrates and products of transamination, especially AS, in the bacterial suspensions. With very short periods of anaerobic incubation the interfering effect of these side reactions is minimized, and it can be shown that reaction  $GL \rightarrow AS$  is extraordinarily rapid in all examined bacterial species. With *E. coli* the optimal pH is 8.5 and the optimal temperature 32° C.; under these conditions and with an incubation time of one minute, the maximal Q value, calculated on the basis of 1 mg. bacterial nitrogen ( $Q_{TN}$ ) was 3900 for *E. coli*. Under less favorable conditions (pH-8.0, 5 minutes' incubation)  $Q_{TN}$  values ranging from 685 (*B. dysenteriae Shiga*) to about 1700 (*Azotobacter*) were found in different bacterial species, including *E. coli*, *E. typhi*, *B. proteus*, *B. pyocyaneus*, *Staph. aureus* and *albus*, *Strept. hemolyt.* and *viridans*, *Pneumococcus Type 1*, *Cl. welchii*. The authors state that the activities of reactions  $GL \rightarrow AL$  and  $AS \rightarrow AL$  are considerably lower in the examined bacteria.

## ✓ V. ROLE OF TRANSAMINATION IN AMINO ACID METABOLISM

### 1. Oxidative Deamination

a. "*l*-Amino Acid Oxidase." Braunstein and Bychkov (29) have pointed out that the properties of the "*l*-amino acid oxidase" of liver and kidney (Krebs, 104) are consistent with the presence of a complex enzyme system capable of deaminating monocarboxylic amino acids indirectly, through (1) transamination with KG, and (2) deamination of the resulting GL by GL-dehydrogenase. This view was supported by the preparation of a cell-free model of "*l*-amino acid oxidase" from gl-apH, GL-dehydrogenase, KG, cozymase, and pyocyanine (or methylene blue). *l*-Alanine was readily oxidized by the complete system with liberation of ammonia.

Braunstein and Azarkh (27, 28) demonstrated that certain *l*-amino acids are actually deaminated in this manner in surviving kidney tissue.

When ground and diluted with saline solutions, pig or rat kidney tissue fails to deaminate *l*-amino acids. In the homogenized suspensions, deamination of GL is restored by the addition of cozymase. When supplemented with cozymase and KG, the homogenates deaminate AS, AL, *l*-cysteic acid, *l*-valine, *l*-leucine and *l*-isoleucine at rates comparable to their rates of deamination in intact kidney slices; deamination in the homogenate is negligible if either cozymase or KG is omitted.

The deamination rates of the amino acids mentioned in supplemented homogenates

(a) and in intact kidney slices (b), and the rates of their transamination with KG (c) decrease in identical sequences, viz.:

GL > AS > AL > Cysteic acid > Valine > Leucine > Isoleucine

All other *l*-amino acids (*l*-serine, *l*-methionine and *l*-phenylalanine were not accessible) were inactive in either of the reactions (a), (b) and (c).

TABLE VIII

*Deamination of l-Amino Acids in Homogenized Pig Kidney Cortex*  
(Braunstein and Azarkh, 27.)

1½–2½ hrs. at 37° C.; KG — 1–2 mg./ml.; Cozymase — 1–2 mg./ml.; dilution of tissues — from 1:7 to 1:40. With 0.01 *M* Na-arsenite.

Substrates	NH <sub>3</sub> formed from added amino acid (average), micromoles per 1 g. tissue				
	Homogenized Tissue				Slices
	Unsupple- mented	With KG	With Cozy- mase	With KG and Cozymase	(Rat Kidney)
GL	5.2	4.3	39.8	28.8	19
AS	2.6	2.6	6.4	23.7	29.5
AL	1.5	2.2	5.5	26.2	19
<i>l</i> -Cysteic acid	0	0	1.5	25.9	—
<i>l</i> -Valine	1.1	1.8	1.2	10.3	7
<i>l</i> -Leucine	0.8	0.6	0.7	8.0	5
<i>l</i> -Isoleucine	—	1.0	2.3	7.1	—
<i>l</i> -Cysteine	6.0	—	—	6.2	8.2
<i>d</i> (—) Threonine, <i>l</i> -Trypto- phan, <i>l</i> -Tyrosine, <i>l</i> -Histi- dine, <i>l</i> -Lysine, <i>l</i> -Proline, <i>l</i> -Hydroxyproline, <i>l</i> -Nor- leucine, <i>l</i> -Kynurenine (31)	0-1.5	—	—	0-2.2	0-3.1

Similar but less clear-cut results were obtained with liver and with kidney tissue of other animals. With a view to the hypothesis of "indirect" deamination here exposed, it is noteworthy that thiamine-deficient rats exhibit parallel decreases of "*l*-amino acid oxidase," and aph activities of liver and kidney tissue (119).

In a paper accessible only in abstract, Cedrangolo and Carandante (53) have apparently obtained results analogous to those of Braunstein and Azarkh. In their opinion, indirect deamination of *l*-amino acids involving transfer of amino-N to KG has not been proven to take part in oxidation of amino acids in the living organism; yet they state that *l*-amino acid oxidase may be identical with gl-aph, at least the two enzymes contain a common component. The latter part of this statement is in full agreement with the mode of deamination discussed above. Cohen (61) obtained negative results in unpublished experiments of a similar kind.

*b. The Fraction of Protein Nitrogen Liberated by GL-Dehydrogenase.* Indirect deamination with the participation of KG certainly is not the only pathway of liberation of ammonia from *l*-amino acids. Suggestive, though inconclusive evidence for the plurality of enzymes oxidizing *l*-amino acids is provided by Edlbacher's studies (70) on the effects of enzyme inhibitors upon deamination of different *l*-amino acids. Green and associates recently isolated specific flavoproteins catalyzing oxidative deamination of glycine (140) and of various *l*-amino acids (82, 15). A peculiar non-oxidative mechanism is involved in the deamination of serine, threonine and cysteine (14, 54, 150).

The physiological importance of the glycine oxidase and *l*-amino acid oxidase of Green is difficult to appreciate at present, because the optimal conditions of their activity (substrate concentration for glycine oxidase, H-ion concentration for *l*-amino acid oxidase) are very remote from those prevailing in living cells, and because the substrates most readily attacked by these enzymes are either poor substrates or altogether resistant with tissue slices or suspensions, and *vice versa*. In the writer's opinion, their discovery does not rule out the suggested mode of indirect deamination. The extent to which the latter mechanism is operative in actual protein catabolism in the living animal cannot be assessed directly. In evaluating its physiological significance, the following facts must be borne in mind.

Many amino acids are subject, in the animal organism, to metabolic transformations resulting in their conversion into GL or other active substrates of transamination (*cf.* Scheme 6, Fig. 2 on p. 36). Some of these changes are irreversible and coexist with reversible deamination of the same amino acids. Amino acids readily convertible to GL and probably catabolized chiefly in this way are — proline, hydroxyproline, histidine, arginine; from phenylalanine and tyrosine AL can be formed (78); cystine is converted in part to cysteic acid (16, 132). Together with AL, AS and GL itself, the sum of these amino acids constitutes no less than 50–65% of the average tissue proteins. The amino nitrogen of some other amino acids is less readily and less completely incorporated in GL.

GL ranks foremost among other amino acids in rapidity of "rejuvenation" of amino-N in the living body (Schoenheimer). The enzymes involved in its formation and breakdown — gl-aph and GL-dehydrogenase — are the most widespread and active enzymes of amino acid metabolism. Formation of GL from other amino acids and its deamination can proceed discontinuously in space and time (in different organs), with eventual temporary stabilization of GL by its inclusion into protein molecules or by the synthesis of glutamine or glutathione.

The inference from these considerations is that, in protein catabolism, probably up to one-half of the amino nitrogen may be ultimately released (as ammonia) by GL-dehydrogenase from GL, either preformed or arising from other amino acids through transamination and other metabolic processes.

## 2. Reductive Amination

*a. Synthesis of Amino Acids in Plants.* From the very first it seemed likely that transamination might play an important part in the synthesis of amino acids in plants, where the dicarboxylic amino acids apparently belong to the first organic products of nitrogen assimilation (34). A complete theory of amino acid synthesis in leguminous plants has been proposed by Virtanen and Laine (161).

They assume the following steps: (1) Reduction of molecular nitrogen to hydroxylamine by the symbiotic nodule bacteria; (2) formation of oximinosuccinic acid through condensation of hydroxylamine with OA formed from carbohydrate in the green plant; (3) reduction of oximinosuccinic acid to AS in the root nodules, and (4) transamination of AS with  $\alpha$ -keto acids to yield amino acids. A few experiments on reaction  $AS \rightarrow AL$  in pea plants were reported (160). Attractive though the theory is, its experimental foundation is very incomplete and far from convincing (*cf.* the discussions by Wilson (168) and Burk (44)).

Euler (74, 1, 2) assumed that enzymatic hydrogenation of imino-glutaric acid and transamination between GL and various  $\alpha$ -keto acids may be of importance as intermediary steps in the synthesis of amino acids from ammonia, both in plants and in the animal organism. Whether the generalizations of Virtanen and Euler are justified, cannot be ascertained until more is known about the substrate specificity of aminopherases in plants.

*b. Formation of Amino Nitrogen in Animal Tissues.* With regard to amino acid synthesis in the animal organism, Euler's hypothesis of the participation of transamination was also quite insufficiently substantiated by experiment. Most recently, Kritzmman and Melik-Sarkissyan (121, 122) have demonstrated that dicarboxylic acids act as primary acceptors and intermediary carriers of  $NH_2-N$  in the formation of amino acids from pyruvate and ammonia in rat liver slices, first described by Neber. (For the sake of brevity, we designate this process conventionally as "reductive amination.") Kritzmman's point of departure was the observation that

TABLE IX  
*Synthesis of Amino Acids by Rat Liver Slices in Ringer-Bicarbonate with  $NH_4$ -Carbonate, and in Ringer-Phosphate with  $NH_4$ -Phosphate (120)*

Added $\alpha$ -keto acid	Amino nitrogen formation (average) $NH_2-N$ in micromoles per 1 g. tissue	
	With $CO_2$	Without $CO_2$
Pyruvic acid	228	24
Oxaloacetic acid	180	180
$\alpha$ -Ketoglutaric acid	35	36

rat liver fails to form amino-N from  $\text{NH}_3$  and pyruvate in the absence of carbonic acid. With OA reductive amination is rapid even in the absence of bicarbonate and  $\text{CO}_2$ , with KG it is slow, but likewise independent of  $\text{CO}_2$  (Table IX).

There is practically no synthesis with  $\alpha$ -ketobutyric or  $\alpha$ -ketoisocaproic acid. Even after a period of incubation in phosphate buffer, the synthesis with PU can be restored by the addition of bicarbonate.

It was inferred that the synthesis from PU involves the intermediary steps:

- (1)  $\text{PU} + \text{CO}_2 \rightarrow \text{OA}$ ; (Wood-Werkman reaction, possibly followed by production of KG through the tricarboxylic acid cycle);
- (2)  $\text{OA (resp. KG)} + \text{NH}_3 + \text{H}_2\text{X} \rightarrow \text{AS (resp. GL)} + \text{X}$ ; (reductive amination of OA, resp. KG);
- (3)  $\text{AS (or GL)} + \text{PU} \rightarrow \text{AL} + \text{OA (or KG)}$ ; (transamination).

This view is supported by the following evidence:

With PU and  $\text{NH}_3$ , — malic acid, AS and GL are formed in addition to AL, the ratio AS/AL being above 1 upon short incubation and falling towards zero in the second hour.

In  $\text{CO}_2$ -free phosphate media the formation of amino acids from PU can be induced by addition of OA or KG or of their metabolic precursors (AS, GL, malic, fumaric, succinic or citric acid), and also, somewhat less efficiently, by tartaric,  $\alpha$ -ketoadipic or mesoxalic acid (*cf.* p. 17). With low ("catalytic") concentrations of added dicarboxylates, as much as 20 moles amino acid are formed per 1 mole dicarboxylic acid. With high ("substrate") concentrations, formation of  $\text{NH}_2\text{-N}$  in the presence of PU is considerably higher than the synthesis without PU, *i.e.*, than the direct reductive amination of dicarboxylic keto acids. In bicarbonate buffer, the synthesis with PU is inhibited, rather than augmented, by added dicarboxylates, presumably through competitive inhibition of enzymes concerned with intermediary transformations of dicarboxylic substrates.

The time relations of AS and AL formation from PU and  $\text{NH}_3$  are much the same in experiments with added fumarate in phosphate buffer as without dicarboxylic acid in bicarbonate buffer (121).

Phosphate is a necessary component of the reaction system (122). With phosphate-free bicarbonate-saline there is no amino acid synthesis, or a very small one, unless catalytic amounts of dicarboxylic acid are added. With borate buffer either a dicarboxylic acid or bicarbonate+phosphate must be added to secure rapid formation of amino-N. These findings indicate the participation of phosphorylation in the Wood-Werkman reaction (*cf.* 129a). The further steps of  $\text{NH}_2\text{-N}$  synthesis do not require added phosphate, though this does not preclude intermediary phosphorylations at the expense of free or organic *intracellular* phosphate.

Preliminary observations with kidney slices point to a similar mechanism of AL synthesis, and it has also been possible to effect an analogous sequence of reactions with chopped heart muscle (Kritzmann, unpublished).

Departing from these results, Kritzmann and Melik-Sarkissyan (123) succeeded in reproducing the amination of PU in cell-free extracts from fresh or acetone-dried liver tissue of various animals. The complete system includes diluted (or dialyzed) liver enzyme extract, ammonium pyruvate, cozymase, small amounts of fumarate or OA, phosphate buffer and some concentrate of co-as-aph. The amounts of  $\text{NH}_2\text{-N}$  synthe-

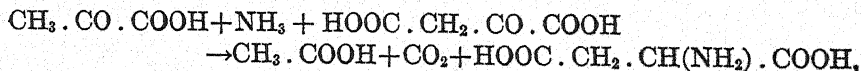
sized in the complete system are sometimes almost as high as in an equivalent amount of liver slices. The rates of  $\text{NH}_2$ -formation in cell-free enzyme systems are parallel, and in fact nearly equal, to the rates of transamination between added AS and PU (Table X).

TABLE X

Substrates	Liver Slices	Liver Extract with Fumarate + Cozymase	Dialyzed Extract + Fumarate + Cozymase	
			Without co-aph	With co-aph
I. $\text{PU} + \text{NH}_2$ ("reductive amination") $\text{NH}_2$ -N formation, micromoles	133	46	0	24
II. $\text{AS} + \text{PU}$ (transamination) AL formation, micromoles	154	45	0	23

It thus appears that dicarboxylic amino and keto acids hold a key position as primary substrates and amino-N carriers in a very active process of amino acid formation ( $Q_{\text{NH}_2\text{-N}} \geq 10$ ) — in fact, the only one proceeding with significant rapidity *in vitro*. This function of the dicarboxylic acids is the counterpart of their similar central position in transamination reactions and in the indirect oxidative deamination of *l*-amino acids. It remains to be ascertained whether the outlined mechanism of amino-N formation is restricted to the synthesis of AS, GL and AL, or plays a broader part in amino acid synthesis in the animal organism.

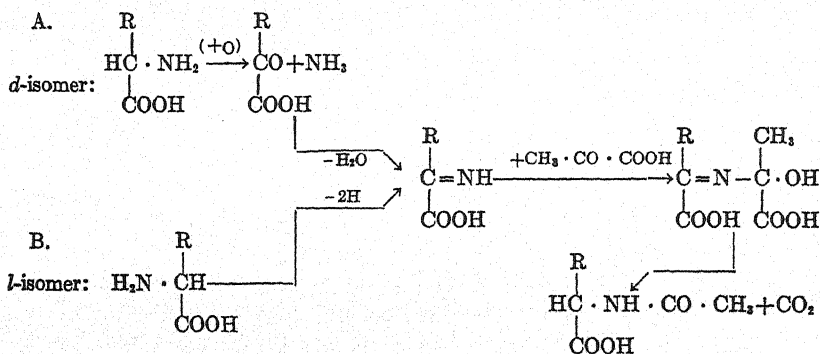
c. "Acetylating Amination." Kritzmann's analysis of the mechanism of reductive amination in liver slices provides a partial explanation for the special role of PU in this process: PU acts as the specific precursor of OA and KG, *i.e.*, of the primary  $\text{NH}_2$ -acceptors. There are probably further reasons for the failure of other monocarboxylic  $\alpha$ -keto acids to replace PU in this reaction. One reason is the high rate of transamination between PU and the originally synthesized amino acids, AS and GL. Apart from this, it seems likely that PU functions as the specific hydrogen donator in the reductive amination of OA and KG. The presumable mechanism is a dismutative reaction:



similar to the dismutation between KG and iminoglutaric acid reported by Krebs and Cohen (110).

An energy-rich form of acetic acid, possibly acetyl-phosphate, would be expected to arise as the primary product of PU oxidation (129a). This might undergo self-condensation to acetoacetate, known to accumulate in liver in the presence of  $\text{NH}_3$  and PU (6, 73), or serve to acetylate available amino groups.

In case of unphysiological aryl- or arylthio-amino acids the resulting N-acetyl compounds are stabilized end products, liable to excretion. In view of established instances of direct amination of preformed amino acids (*e.g.*, mercapturic acid syntheses) this mechanism seems more probable than acetylating reductive amination according to the theory of Knoop and du Vigneaud. In the latter theory, PU acts *simultaneously* as hydrogen donor and acetylating agent. On the basis of their tracer experiments with rats receiving  $\text{D}_2\text{O}$  and  $\alpha$ - or  $L$ - $\text{N}^{15}$ -phenylaminobutyric acid, du Vigneaud and associates (159) suggested a mechanism of acetylation involving condensation of PU with imino acids and intramolecular reduction and acetylation of the imino group (Scheme 5).



Scheme 5

The mode of reaction (B) was assumed especially in order to account for the uptake of deuterium, with no exchange of amino nitrogen, in the  $\alpha$ -position of the N-acetyl derivative formed from  $L$ - $\text{N}^{15}$ -phenylaminobutyric acid. An alternative explanation of this phenomenon, suggested by the authors, is an exchange of  $\alpha$ -hydrogen in the course of transamination reactions, followed by direct acetylation. The interconversion of Schiff's bases during the course of enzymatic transamination is, in fact, a prototropic change involving replacement of the  $\alpha$ -hydrogen (see p. 7). To this path of reaction it might be objected that aromatic amino acids are inactive in enzymatic transamination *in vitro*, but this argument may not be valid with regard to the living organism. Moreover, it appears from our recent experiments with deuterium-labeled amino acids (p. 7)\* that, in many instances, the  $\alpha$ -hydrogen of an amino acid can undergo labilization and dissociation in the presence of aminophenase, even though the conditions are such that no electromeric shift resulting in actual transamination ensues. Therefore, replacement of  $\alpha$ -hydrogen in amino acids without exchange of nitrogen does not necessarily implicate intermediary dehydrogenation at the  $\alpha$ -carbon atom. This interpretation would also apply to other instances of similar  $\alpha$ -hydrogen replacement, *viz.*, in the studies of Bovernick (19) on bacterial synthesis of *d*-GL polypeptides. The experiments of Bernhard (13a) and of Bloch and Rittenberg (16a) with labeled acetate support the view that the biological acetylation of amino acids proceeds chiefly through direct conjugation with acetate, rather than through "reductive acetylation" with PU.

\*See also Addendum 1, p. 52a.

### 3. *Interconversion of Amino Acids and "Rejuvenation" of Protein Nitrogen*

The brilliant isotope studies of Schoenheimer and coworkers have revealed a strikingly active shifting of amino-N between all amino acids (lysine excepted) of the tissue proteins in the living animal, as evidenced by the rapid uptake of  $N^{15}$ , ingested in the form of ammonia or of various *d*- or *l*-amino acids. The highest content of  $N^{15}$  is invariably found in the dicarboxylic amino acids, especially in GL. The same holds true for the distribution of  $N^{15}$  in the amino acids from the proteins of plants nourished with isotopic nitrogen compounds (158), or of *Azotobacter* after a short period of assimilation of  $N_2^{15}$  (45). This is usually taken to indicate the importance of transamination in the redistribution and rejuvenation of amino nitrogen (147), although the evidence is by no means unequivocal. In many cases, *e.g.*, upon ingestion of  $N^{15}$ -*d*-amino acids, intermediary liberation of ammonia is indubitable. The ready availability of KG and OA as intermediates of carbohydrate metabolism and the rapidity of their reversible amination is in good accord with a key position of GL and AS in the redistribution of amino-N (147). However, the possibility of an independent and rapid individual turnover of the aminodicarboxylic acids affords an alternative explanation for the preferential renewal of  $NH_2$ -N in the GL and AS of tissue proteins.

For the present, the conception of a general function of transamination in the rejuvenation of protein nitrogen *in vivo*, with GL and AS acting as intermediary  $NH_2$ -carriers, is opposed by the restricted substrate specificity of aminopherases *in surviving tissues*. It should be stressed, however, that there is also a similar disparity between the living organism and surviving tissues or enzyme extracts with regard to the range of substrates available for reductive amination or oxidative deamination. *In vivo*, all *l*-amino acids are readily deaminated, and most are synthesized from their respective  $\alpha$ -keto analogs, but in sliced or minced tissues very few *l*-amino acids undergo appreciable deamination, whilst reductive amination has thus far been achieved only with PU, OA and KG. It well may be that the scope of transamination is likewise broader *in vivo*, and that the choice of substrates active *in vitro* is narrowed by the failure to secure optimal experimental conditions. These considerations must be kept in mind in assessing the possible biological implications of the experimental findings on transamination discussed in this review.

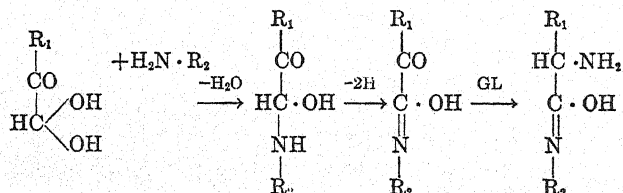
### 4. *Protein Synthesis*

No definite relationship has yet been established between aph activity and the rates of protein synthesis in growing or stationary tissues. Transamination is slow in many secretory, incretory and proliferative organs capable of rapid protein synthesis (24). Cohen (61, 63) regards the low aph



activity of rapidly growing embryonic and malignant tissues as evidence of an inverse relation between the rates of transamination and of protein synthesis. He assumes that transamination may control protein synthesis by the rapid removal of GL. Since transamination is an equilibrium reaction, the argument can be reversed, *i.e.*, high aminopherase activity may ensure the rapid formation and utilization of GL for the synthesis of protein. In a paper of later date, Cohen himself, in collaboration with Albaum (5), takes this opposite standpoint with respect to the protein metabolism of germinating oat seedlings, and emphasizes the parallel increases of aph activity, of non-protein N and rate of protein formation. According to Shmerling (149) there is no direct or inverse correlation between gl-aph activity and alterations of the growth constants of individual organs at different stages of development, in the rabbit embryo.

In keeping with the general trend of modern theories of biological synthesis, mechanisms of protein synthesis other than the reversal of enzymatic proteolysis have recently been suggested. Linderström-Lang (129) has proposed the following speculative scheme of peptide synthesis:



*i.e.*, (1) formation of hydrated Schiff's bases from glyoxals and amino acids, (2) dehydrogenation of the condensation products, and (3) transamination of GL with the resulting  $\alpha$ -ketoacylamino acids to yield peptides. There is nothing to indicate the biological occurrence of reactions like (1) or (2). Herbst and Shemin (94), reporting non-enzymatic transamination with ketoacylamino acids, discuss the possible occurrence of reaction (3) as an intermediary step in biological peptide synthesis. As stated above (p. 18) the only experimental observations of enzymatic transamination with peptides and ketoacylamino acids, reported by Ågren (4), are not reliable.

## VI. INTEGRATIVE FUNCTIONS OF THE DICARBOXYLIC ACIDS IN NITROGEN METABOLISM

### 1. Chemical Integration in Nitrogen Metabolism

*a. The Regulation and Coordination of Metabolic Processes.* A major function and characteristic feature of metabolism in animals is the maintenance of approximate qualitative and quantitative constancy of the chemical constituents of tissues and body fluids at rest, and restitution of their stationary composition and concentrations following alterations due to functional activity or adaptive response. Stability of the chemical pattern

of living organisms is based on coordination of metabolic processes by the complicated interplay between neural and humoral regulations and the automatic control of intracellular reactions. Like other metabolic processes, the metabolism of nitrogen, *i.e.*, the sum of biochemical transformations of proteins, amino acids and their proximate and remote derivatives, is more or less rigidly controlled.

Under normal resting conditions only narrowly limited variations occur in the composition and concentrations of the proteins and various non-protein nitrogen fractions of body fluids and tissues. The same holds true for the rates of production and removal of specialized, biologically active protein derivatives — proteinogenous amines, hormones, mediators, and the like. In functional activity and adaptive reactions, these rates are altered in exactly regulated proportions. The extent of formation, destruction and elimination of intermediates and end products of protein metabolism automatically adjusts itself to widely different levels of protein intake, securing maintenance of nitrogen equilibrium. The absolute amounts of excreted nitrogen compounds are, therefore, variable, yet the percentage ratios of urinary end products — ammonia, urea, amino acids, *etc.*, are fairly constant.

The recent isotope work of Schoenheimer and others has demonstrated that an exceptionally rapid, incessant replacement of nitrogen and other structural elements takes place in all nitrogenous tissue constituents, even those formerly thought to be most stable—protoplasmic proteins, nucleic acids, phospholipids of brain and other tissues. This rapid turnover indicates a continuous alternation of processes of profound cleavage, inter-conversion, resynthesis and total decay. The stationary condition of high- and low-molecular tissue components necessitates an astonishingly harmonious coördination of these processes. An important outcome of the isotopic researches is the proof that there exists no independent endogenous and exogenous metabolism of the proteins, purines, creatine or other nitrogen compounds. Moreover, the incessant shifts and transformations of nitrogenous groups and other structural elements (methyl or sulf-hydryl groups, carbon chains) are not confined within the limits of one individual class of compounds or another. The structural units merge into a common "metabolic pool" (Schoenheimer), whence they are drawn upon for the resynthesis of compounds of all classes, in proportions commanded by the velocities of automatic reactions of cell metabolism and by the influence of regulatory mechanisms.

*b. Integrative Components in Nitrogen Metabolism.* A distinctive feature of protein metabolism, as compared to the metabolism of fats or carbohydrates, is the multiplicity of its components and its exceedingly complex branching. Nitrogen metabolism in the animal organism involves several hundred derivatives, intermediates and end products originating from twenty-odd amino acids of the protein molecule. The number of enzymes and other biocatalysts engaged in their transformations also probably

exceeds one or two hundred. This peculiarity opposes special difficulties to the investigation and interpretation of coordination in nitrogen metabolism, and of the controlling mechanisms involved.

Separate existence and independent regulation of a great number of topographically scattered and multifarious chemical transformations would be incompatible with the perfect balance of dissimilation and assimilation of nitrogenous constituents, their steady levels in cells and biological fluids, with the rapid redistribution of isotopic nitrogen — whatever the ingested form — in constant proportions between the nitrogenous tissue constituents, and with other features of correlation of all aspects of nitrogen metabolism. The harmonious physiological integration of a multiple system of processes of such complexity can only be understood on assumption of a few central metabolic links, bearing a relation to considerable groups of these processes, directing them into common channels in certain parts of their course, and serving as the main points of application of regulatory and controlling factors. Such central metabolic components and the biocatalysts involved in their transformations act as collectors of nitrogenous material converging from different streams into common courses, as sluiceways and storage basins, as conduits and dispensing devices, by means of which the nitrogenous material is further distributed over a ramified system of channels. They hold the cross-roads connecting nitrogen metabolism with tissue respiration and with the metabolism of fats and carbohydrates, and control the interchange of intermediates. Along with these central links of general coordination — we may term them *integrative components of first order* — there exist *subordinate integrative components of lower order*, controlling and unifying special groups of metabolic processes of narrower scope. Examples of subordinate integrative system in nitrogen metabolism are the amidine system, comprising arginine with its derivatives and pertinent enzyme systems and commanding the metabolism of urea and creatine, or the systems of methyl and sulfhydryl transfer (methionine-choline, homocysteine-cysteine, and their respective enzyme systems), connected with each other and with the amidine system by common components.

The collector, shunting and distributive functions of the integrative links of metabolic coordination partly coincide with the similar functions of polyenzymatic coenzyme systems as defined by J. K. Parnas (137). Among these, in the first place, we must look for integrative components of metabolism.

c. *The Dicarboxylic Acid System.* At the present level of knowledge it would be premature to attempt a complete description of the nature, interconnections and hierarchy of all integrative components of nitrogen metabolism. However, the work of many biochemists in different countries

has brought together, in the last decade, a large body of evidence definitely pointing to the primary and very general importance of one system of compounds in the chemical integration of nitrogen metabolism. This central integrative system of first order, characterized by the multiplicity of its cross relations, inflows and outlets all over the metabolic pathways of nitrogen, is the *system of the dicarboxylic amino acids*. We can define it as comprising GL and AS, their amides, the nitrogen-free di- and tricarboxylic acids connected to them by reversible or cyclic interconversions, and the enzymes and coenzymes taking part in the transformations of these acids; the system also includes AL and PU, linked to the dicarboxylic acids by intimate metabolic relations. Some of the functions of GL, AS and AL in the formation, transfer and breakdown of amino groups have been discussed in the preceding parts of this review. In the present chapter, their role in other reactions of nitrogen compounds and their general functions in metabolic coordination in the animal organism are considered. A few years ago, the analogous functions of the dicarboxylic acid system in plants were discussed in detail by Chibnall, in an excellent monograph (55) to which the reader is referred.

## 2. *Metabolic Sources and Mutual Relations of the Amino Dicarboxylic Acids*

Free AS and GL and glutamine are regular constituents of the non-protein nitrogen fraction of tissues and blood; they account for  $\frac{1}{4}$  to  $\frac{1}{6}$  of the  $\alpha$ -amino-N of this fraction. Large amounts of GL and AS are continuously liberated by the enzymatic hydrolysis of tissue and food proteins, which frequently contain as much as 25% dicarboxylic amino acids, or more. Many other amino acids are transformed into components of the dicarboxylic acid system by special metabolic reactions.

Glutamine is formed from histidine, *via* formyl-glutamine, by the action of histidase (69) and also in an alternative series of catabolic reactions through urocanic acid as intermediate (71). That GL is formed in the course of oxidation of proline and hydroxyproline and of ornithine, has been demonstrated both in experiments with tissue slices (167, 105, 106) and in intravital studies with labeled amino acids (141, 152). Metabolic formation of a dicarboxylic acid, possibly GL (*cf.* p. 40), from lysine is indicated by the capacity of lysine to replace GL or AS in the conversion of citrulline to arginine (Borsook and Dubnoff, 17). The oxidation of tyrosine (and consequently, of phenylalanine) in liver gives rise to AL (78, 39). Cysteic acid, a further dibasic acid active in transamination, is a physiological intermediate in the oxidation of cystine. PU is a product of the anoxidative cleavage of cysteine (14, 150) and of serine (14, 54).

In Fig. 2 a scheme is given for the genetic interrelations of amino acids, converging toward components of the dicarboxylic acid system. The scheme includes all amino acids with the exception of valine, leucine and isoleucine, capable of slow transamination, and of glycine and tryptophan, which are catabolized by independent pathways.



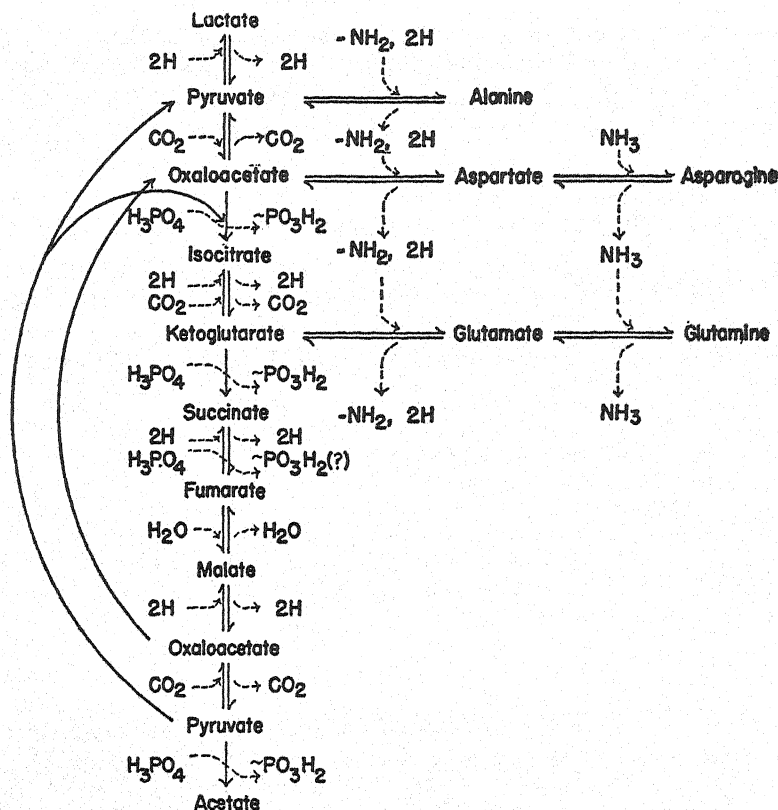


Fig. 3. Scheme 7. Interconversions of the components of the dicarboxylic acid system, and their acceptor and carrier functions.

are reversible reactions in which dicarboxylic acids act as typical coenzymes, or intermediary carriers of certain chemical groups. Thus, the reactions  $OA \rightleftharpoons malate$  and  $fumarate \rightleftharpoons succinate$  are reversible catalytic links in the respiratory transport of hydrogen; the pairs  $GL/KG$ ,  $AS/OA$  and  $AL/PU$  transfer amino groups in transamination reactions. Since the dicarboxylic carriers can be connected to more or less varied antecedent donors or ulterior acceptors by means of different enzyme systems, they act in the manner of polenzymatic coenzymes according to Parnas (137).

Of analogous metabolic significance are cyclic chains of transformations ending in regeneration of catalytically active components, for example, the Krebs cycle as a whole: here  $OA$  "accepts"  $PU$  (or an active acetate residue) by condensing with it to isocitrate, and is reconstituted in a chain of reactions, the net result of which is the complete oxidation of  $PU$ . Important energy-absorbing synthetic steps are associated with the cyclic

transformations of dicarboxylic acids. In these reactions inert inorganic end products of metabolism are reincorporated into organic compounds as active structural elements with more or less elevated group potential, acquired at the expense of "coupled" inter- or intramolecular exergonic changes. In these syntheses, dicarboxylic acids function as transformers of chemical energy and as acceptors or intermediary carriers of the newly formed metabolically active groups.

Thus, the oxidations:  $OA + PU \rightarrow$  Isocitrate,  $KG \rightarrow$  Succinate,  $PU \rightarrow$  Acetate, and probably  $Succinate \rightarrow$  Fumarate, are associated with aerobic phosphorylation, inorganic phosphate being primarily transformed into energy-rich phosphate groups of intermediary acyl-phosphates, and further transferred to the adenylic system or other acceptors. In the Wood-Werkman reaction, inorganic  $CO_2$  is fixed as organic carboxyl in OA, energy being probably supplied by phosphorylation reactions. A similar process is the reversible reaction:  $Oxalosuccinate \rightleftharpoons KG + CO_2$ , recently discovered by Ochoa (1933a). In the reductive amination of OA and KG, energy provided with hydrogen from unidentified donors serves to transform ammonia into  $\alpha$ -amino groups available for transamination and other synthetic reactions (17). Respiratory and glycolytic oxidoreductions are linked by an unexplored mechanism of energy transfer with formation of the amide groups of glutamine (and, in plants, of asparagine) from ammonia; these amide groups appear to be utilized in metabolism, in preference to free ammonia, for the synthesis of different nitrogen compounds.

Each conversion of a dicarboxylic acid into another link of the cycle automatically blocks certain acceptor or carrier functions and opens other metabolic circuits.

Thus, the reaction  $OA \rightarrow$  Malate brings into action catalytic hydrogen transfer and blocks reversible decarboxylation and amino nitrogen transfer. Formation of GL from KG opens the way for amidation, while it renders the dicarboxylate molecule inactive in  $CO_2$  fixation or oxidative phosphorylation and interferes with the Krebs cycle. The conversion of GL to glutamine or of AS to asparagine stops transamination, and so on, all these interrelations being reciprocal.

The implications with respect to metabolic coordination are self-evident. The shunting effects of shifts in the dicarboxylic acid system afford exceedingly manifold possibilities for the mutual automatic control of various phases of nitrogen metabolism and cellular respiration and for their regulation by humoral and neural factors. The relative and absolute rates of anabolic and catabolic reactions in nitrogen metabolism will largely depend on the state of the dicarboxylic acid system, *i.e.*, on activation and inhibition of the pertinent enzyme systems, on the supply or removal of coenzymatic carriers of hydrogen and labile phosphate and of intermediates in carbohydrate oxidation, on the amounts of available ammonia and active amino or amide groups.

#### 4. *Reciprocal Regulations of Cellular Oxido-Reductions, Amino Acid Metabolism and Acid-Base Economy*

The importance of transamination in regulating cellular respiration by the removal of dibasic C<sub>4</sub>- and C<sub>5</sub>-keto acids, acting as respiratory catalysts in Szent-Györgyi's system of hydrogen transport and in the Krebs cycle, was discussed in the first review on transamination (Braunstein, 24). This aspect of the functions of transamination has since been somewhat one-sidedly emphasized by Martius (131) and by Cohen (59, 61), who tends to disregard the immediate relations of this process to nitrogen metabolism.

Anaerobic glycolysis in brain (166) and in muscle is inhibited by GL, because the conversion of PU to AL by transamination interferes with glycolytic oxido-reduction and phosphorylation (Grodzensky, 83).

In evaluating the reciprocal influence of carbohydrate oxidation and amino acid metabolism it must be borne in mind that the individual metabolic functions of PU, OA or KG, and of AL, AS or GL, are quite different and specific. Their rapid mutual replacement in transaminations will not only affect the rates of metabolic processes, but also qualitatively alter their direction.

For instance, shifts in the equilibria  $AS + PU \rightleftharpoons AL + OA$  and  $GL + PU \rightleftharpoons AL + KG$  will be interdependent with the prevailing trend for liberation of CO<sub>2</sub> from OA, or its fixation in the Wood-Werkman reaction and subsequent stabilization as AS (resp. CO<sub>2</sub> fixation in Ochoa's reaction or stabilization of KG, in the form of GL). Only KG, but not OA, is active in catalyzing indirect deamination (p. 24), whereas the observations of Kritzmann and Melik-Sarkissyan seem to indicate that chiefly OA, rather than KG, is the primary acceptor of ammonia in reductive amination. On the other hand, in animal tissues only GL is available for the reversible binding of ammonia as amide, while AS is inactive.

These examples point to the existence of important mutual relations between the interconversions of C<sub>4</sub>- and C<sub>5</sub>-dicarboxylic acids by transamination or in the Krebs cycle, on the one hand, and the binding of CO<sub>2</sub> and of ammonia or their release, pertaining to the regulation of acid-base equilibrium, on the other hand (cf. p. 41).

#### 5. *Role of the Dicarboxylic Acid System in Gluconeogenesis*

The formation of carbohydrate from protein in the animal body is intimately connected with the dicarboxylic acid system. As pointed out by Krebs (109), chiefly those amino acids are glucogenic which undergo oxidation to ketonic acids of the tricarboxylic cycle, either directly or after preliminary transformation into GL. Krebs enumerates eight such amino acids: AL, AS, GL, histidine, proline, hydroxyproline, arginine and citrulline. Tyrosine, forming PU through AL (78), is also glucogenic (47); the same is true of phenylalanine (46). As stated earlier in this review (p. 25) deamination of all these amino acids proceeds with the participation of GL-dehydrogenase and, eventually, of aminophosphatases. Their sum



accounts for more than half of the protein molecule. Further glucogenic amino acids are threonine (85), serine and valine, forming PU by reactions bearing no relation to the dicarboxylic acid system. The glucogenic activity of cystine is controversial, although its enzymatic conversion to PU is an established reaction (150, 14). In good agreement with the known mechanisms of conversion to PU, only three carbon atoms of each of the glucogenic amino acids are utilized in carbohydrate formation, irrespective of the total number of carbon atoms in the original molecule.

The antiketogenic activity of the glucogenic amino acids seems to be more directly connected with their combustion through the tricarboxylic acid cycle than with replenishment of carbohydrate stores.

#### 6. *The Conversion of Citrulline to Arginine*

An important reaction relating the aminodicarboxylic acids to the metabolism of creatine and of urea is the rapid synthesis of the amidine group of arginine through interaction of GL or AS with citrulline in the kidney (Borsook and Dubnoff, 17). In this reaction, requiring the integrity of cell structure and inhibited by oxidation inhibitors (cyanide,  $As_2O_3$ ,  $As_2O_5$ ), GL and AS are equally active as donors of the imino group of arginine. They can be replaced by  $KG + NH_3$ , or  $OA + NH_3$ , and by ornithine, proline or hydroxyproline, undergoing conversion to GL in kidney slices. Lysine is also active — a fact taken by Borsook and Dubnoff to indicate its transformation into GL; in view of the activity of  $\alpha$ -ketoadipic acid in transamination (p. 17) and in reductive amination (p. 28) the structurally plausible intermediary formation of  $\alpha$ -amino adipic acid from lysine seems more likely.

The reaction is a source of arginine required for the formation of guanidoacetic acid by transamidination with glycine in the kidney. The source of citrulline in the kidney is unknown. It is remarkable that the interaction of citrulline with AS or GL to form arginine apparently does not occur in liver, although conversion of citrulline to arginine is a highly probable intermediary step in the ornithine cycle of urea formation (*cf.* Gornall and Hunter, 80).

#### 7. *Metabolism and Functions of Glutamine and Asparagine*

In the protein molecule, GL and AS are present chiefly in the form of their amides. Free asparagine and glutamine are common constituents of plant tissues, being actively synthesized in germinating seeds and sprouts, and stored in large amounts in the leaves and roots of some species. More recently, it has been recognized that glutamine is readily synthesized from GL or KG and  $NH_3$ , in mammalian organs, and that specific amide-hydrolyzing enzymes — glutaminase and asparaginase — are present in

varying amounts in animal tissues (105). Free glutamine occurs in significant quantities in most organs and in blood (78a, 86, 86a, 87, 7), representing an important source of traumatic, resp. postmortal, ammonia formation, while asparagine has not hitherto been detected in animals.<sup>1</sup>

The widespread occurrence of the dicarboxylic amides, their active metabolism and their importance as essential nutrients, or growth stimulators, for various microorganisms has aroused considerable interest in their biological significance.

*a. Detoxication, Transport and Excretion of Ammonia.* One of the earliest and most widely accepted views of the role of asparagine in plants was the hypothesis of Boussingault, further developed by Prianischnikow (*e.g.*, 138) that asparagine formation is a result of respiratory combustion of proteins in darkened plant tissues, similar to the production of urea in the animal, from which it differs in that asparagine can again be utilized in the plant for protein synthesis. In Prianischnikow's opinion, amide formation is a mechanism for the detoxication of ammonia. That this is the only or principal purpose of amide synthesis in the plant, can no longer be maintained in the light of recent phytochemical investigations (*cf.* Vickery, 157, 156; Chibnall, 55). In animal metabolism, however, the rapid removal of ammonia at the sites of its metabolic formation is doubtless a very important function of glutamine synthesis.

Animal tissues are more susceptible than plants to the toxic action of ammonia and would not tolerate its accumulation to any significant extent. The nervous system is especially sensitive, ammonia acting as a potent excitatory and convulsive poison. Liberation of ammonia from various sources (probably including glutamine) takes place in the nerve cells of brain and retina and in peripheral nerves, especially during activity. The rapid synthesis of glutamine in brain and retina, with utilization of energy supplied by respiration or glycolysis, is capable of "mopping up" any ammonia formed, thus preventing its accumulation. It is possible that liberation of ammonia plays a physiological part in excitation, and its removal by glutamine synthesis in the recovery of the nerve cells in brain and retina.

The detoxifying action of GL is strikingly demonstrated by Sapirstein's (145) observation that GL is highly efficient as an antidote for the central action of injected ammonium salts, and by the clinical studies of Price, Waelsch and Putnam (139) on the favorable effect of GL in attacks of epileptic *petit mal*.

As a catabolic waste product ammonia must be translocated from the different tissues to liver for conversion into urea, and to the kidneys for excretion. Circulating blood, however, contains practically no free ammonia (Parnas, Conway). Glutamine, present in blood plasma in concentrations of 5-12 mg.% (86, 87, 7), acts as the vehicle of ammonia.

Conspicuous evidence for the function of glutamine as a transport form and reserve of ammonia and, incidentally, for its role in the regulation of acid-base economy (see p. 39) is provided by the recent studies of Van Slyke and collaborators (155). These

<sup>1</sup>A unique exception is the recent isolation of asparagine from the hemolymph of the larvae of *Melolontha* (Ussing, 154b).

authors showed that glutamine is the chief source of urinary ammonia. In experimental acidosis, more glutamine is withdrawn from the blood on its passage through the kidney, to supply additional ammonia for neutralization of the excreted acids; in alkalosis the formation of ammonia in the kidney from blood glutamine is diminished.

*b. Recapture and Storage of Protein Nitrogen.* In the respiration of plant organs deficient in carbohydrate (carbohydrate-poor germinating seeds, developing leaf buds, etiolated sprouts, darkened leaves) large amounts of protein are oxidized with the simultaneous synthesis of dicarboxylic amides. In the amide and amino groups of each molecule of asparagine or glutamine, two atoms of nitrogen are provisorily laid in store in this way, in a mobile and metabolically active form. This nitrogen is readily available for subsequent regeneration of protein, when an adequate supply of carbohydrate for energetic and plastic purposes is provided by photosynthesis or in some other way.

Glutamine synthesis in the animal connotes a similar recapture and interim storage of amino-N from amino acids undergoing catabolic breakdown, and of ammonia from different sources. It is immaterial whether the amino group of the glutamine has been formed by reductive amination or transamination of KG, or otherwise. What is important from the standpoint of metabolic control, is the stabilizing action of amidation with regard to the amino group, protected from transamination, and probably from deamination and other changes, so long as the amide group is in place. Unlike the nitrogen of the end products urea, creatinine, uric acid or allantoin, the amide nitrogen of glutamine is available for various metabolic syntheses in the animal body, and its removal unblocks the similarly active amino group of GL. There is good reason to consider glutamine as one of the major undifferentiated, non-specific constituents of Schoenheimer's generalized "metabolic pool" of nitrogen.

*c. Utilization of Glutamine in the Metabolic Synthesis of Different Nitrogenous Compounds.* In the sea-urchin egg, onset of development following fertilization or chemical stimulation induces a rapid liberation of ammonia, associated with abundant formation of glutamine, these changes being coincident with the active reconstruction of nitrogenous cell constituents (Ørstrøm, 134). The rates of enzymatic hydrolysis of glutamine are equal in unfertilized and fertilized eggs, but only in the latter is glutamine rapidly synthesized from GL and ammonia, reaction:  $GL + NH_3 \rightleftharpoons \text{glutamine}$  being in equilibrium at 80-85% glutamine. The fertilized egg actively absorbs  $NH_3$  and GL from the aqueous medium, the storage of ammonia being increased in the presence of GL. The phenomena observed by Ørstrøm in the fertilized sea-urchin egg bear considerable likeness to the amide metabolism in germinating seedlings, and suggest that dicarboxylic acid amides may act as immediate sources of nitrogen for the synthesis of essen-

tial nitrogenous components of protoplasm, in plant and animal cells alike. A detailed study of amide metabolism in the embryonic development of warm-blooded animals might prove of considerable interest, as indicated by the fact that the yolk of hen's egg is rich in glutamine (35 mg.%) in comparison to other animal objects (7).

A special function of the dicarboxylic amides in the synthesis of urea is postulated by Leuthardt (126), who found that urea is more readily formed in liver slices from glutamine or asparagine than from ammonia. He concludes from his experimental data that the ornithine cycle is not operative in the conversion of glutamine to urea, and postulates a direct transfer of amide nitrogen ("transamidation"). No catalytic function of glutamine as carrier of amide groups could be demonstrated (cf. Borsook and Dubnoff, 18).

S. J. Bach (9), on the basis of unconvincing experimental data, assumed such a carrier function of glutamine, and further, attempted to combine Leuthardt's views on urea formation with the ornithine cycle of Krebs, by suggesting a highly improbable scheme of oxidation of citrulline to GL+urea. The role of glutamine in urea formation has been challenged by Krebs (108), who found that ammonium glutamate is as active as glutamine in promoting the synthesis of urea, and severely criticized the experiments of Bach. An unsuspected source of error in studies on urea formation from glutamine, revealed by Archibald (7a), is the regular presence of arginine as a contaminant in ordinary preparations of glutamine. Still the question at issue cannot be considered as settled, especially in view of new data by Leuthardt and Glasson (128) indicating that succinamide is a more efficient precursor of urea than ammonium succinate. F. and M. Bernheim (13b) report that the synthesis of urea from  $\text{NH}_3$  in liver slices can be inhibited by caffeine and restored by the addition of either ornithine or glutamine.

Ørstrøm, Ørstrøm and Krebs (135) demonstrated that the synthesis in pigeon liver of hypoxanthine — the metabolic precursor of uric acid — is greatly promoted by glutamine, either alone or especially in the presence of OA or PU. The increase of hypoxanthine formation by  $\text{NH}_3$ +OA (or by  $\text{NH}_3$ +PU in bicarbonate buffer) is due to the fact that glutamine is readily synthesized in pigeon liver slices from the mentioned precursors (Ørstrøm *et al.*, 136). These data indicate that the amide group of glutamine, rather than free ammonia, may act as the direct source of the nitrogen of uric acid in birds.

A comparison of the findings of Ørstrom, Ørstrom and Krebs with the results of isotope studies by Barnes and Schoenheimer (11) suggests the possibility that the purine and pyrimidine bases of nucleic acids in birds and mammals may be synthesized in the same manner. In the experiments of Barnes and Schoenheimer, the  $\text{N}^{15}$  of ingested isotopic ammonia was rapidly incorporated in the purines and pyrimidines of internal organs, both in the pigeon and the rat. The isotope concentrations were almost equal in the ring nitrogen of either purines or pyrimidines, and in the amino groups of guanine and adenine; the uric acid excreted by the pigeon contained almost three times as much  $\text{N}^{15}$ , and the allantoin from rat urine about the same concentration as the respective

nucleic acid bases. It is perhaps no mere coincidence that the isotope concentration in the amide nitrogen of the visceral proteins was nearly the same as in the tissue purines and pyrimidines.

In slices of guinea pig liver, incubated with glutamine and benzoic acid, Leuthardt (127) observed the formation of significant amounts of hippuric acid, which was isolated and identified (only a slight synthesis was obtained with rat liver slices). In minced brain (105) and heart (78a) glutamine disappears without the formation of ammonia.

Beyond the few mentioned facts, little positive information is available on the direct utilization of glutamine in animals. It is tempting, however, to venture into some speculation on this point.

The presence in both glutamine and glutathione of a CO-NH linkage in the  $\gamma$ -position, rarely encountered elsewhere, and the rapid formation of both compounds in liver, point to possible interrelations and suggest the idea that an exchange reaction or condensation of the amide group of glutamine might be involved in the synthesis of glutathione. The ease with which the  $\gamma$ -amide linkage enters into exchange reactions is illustrated by the ready formation of pyrrolidone carboxylic acid from glutamine *in vitro* and by its production during the enzymatic hydrolysis of glutathione (Woodward and Reinhard, 169). The possibility that pyrrolidone-carboxylic acid is an intermediate in the biological synthesis of proline, and indirectly of ornithine, cannot be dismissed for the present, despite the lack of positive evidence.

Glutamine and asparagine have been shown to act as growth substances for yeast and certain bacteria (Nielssen, McIlwain). Their effect is possibly explained by the observations of Bovarnick, who showed that nicotinic acid amide can be replaced, in nutrient media for microbes incapable of synthesizing this vitamin, by a heated mixture of asparagine and GL (20), and that nicotinic acid amide can actually be isolated from such a mixture (21). It is highly interesting whether nicotinic acid can be synthesized biologically from the same precursors in those microorganisms and animals (*e.g.*, rats) which can dispense with an exogenous supply of nicotinic acid.

There is one further important question awaiting experimental solution: can the amide groups linked in  $\gamma$ -position to the dicarboxylic amino acid residues in the tissue proteins directly participate in metabolic reactions without preliminary liberation as free glutamine or asparagine? That this may be the case is indicated by the very high rate of incorporation of ingested isotopic nitrogen in the amide groups of tissue proteins. In the liver proteins, the isotope content of the amide nitrogen considerably exceeds that of any other constituent, including the amino group of GL. Only the amidine group of arginine attains a similar excess of  $N^{15}$ , which is almost as high as the isotope content of urinary ammonia or urea at the end of the experimental period (147, 148).

#### 8. *Significance of the Dicarboxylic Acid System in Some General Disturbances of Protein Metabolism*

Little is known at present on the mode of action of hormones and other humoral agents and of neural mechanisms in regulating nitrogen metabolism, and on their primary points of attack. With a view to the integrative functions of the dicarboxylic acid system, it is of interest to point out a few instances where disturbed regulations of protein metabolism can be

traced to imbalance of mechanisms more or less directly affecting transformations of components of this integrative system.

In a number of pathological conditions, the catabolism of body proteins is increased, tending to compensate by augmented glyconeogenesis, or by immediate respiratory combustion, for deficiency, excessive breakdown or faulty utilization of carbohydrate.

Thus, the general impairment of carbohydrate metabolism in nutritional thiamine deficiency is associated with negative protein balance (Lävroff and Yarussova, 124), with increased urinary excretion of keto acids and total, urea and amino N (Kritzmann, 119), the ratio of urea N to total N being lowered. Similar disturbances of nitrogen metabolism are observed in endogenous B<sub>1</sub>-hypovitaminosis attending traumatic lesions of the nervous system (Kaplansky, 95) or experimental painful stimulation of peripheral nerves (Braunstein, Kritzmann and Azarkh, 39). The underlying failures of intermediary metabolism, due to the lowered content of diphosphothiamine enzymes in the tissues, are: decreased rate of oxidation and condensations of  $\alpha$ -keto acids in the Krebs cycle (Barron and associates, 12) and inhibition of transamination, *l*-amino acid deamination and especially of reductive amination (118, 119). The marked increase of arginase (68, 72) and of histidase (72) in the liver of thiamine-deficient animals is considered by Edlbacher (72) as an adaptive mechanism serving to provide additional GL and KG in compensation for their inadequate formation from carbohydrate.

The well-known increase of gluconeogenesis from protein in human and experimental diabetes is likewise connected with depletion of carbohydrate stores in the tissues. It is significant in this respect that the only immediate effects of insulin upon intermediary metabolism so far established *in vitro* are concerned with components of the dicarboxylic acid system. These effects are: the increase by insulin of the rate of citrate oxidation in muscle tissue (Krebs and Eggleston, 111), and its inhibitory action upon oxidative deamination of AL, AS and GL in liver slices (Bach and Holmes, 9; cf. Stadie, Lukens and Zapp, 151).

Adrenal cortical deficiency involves an impairment of gluconeogenesis from amino acids (Long, Katzin and Fry, 130) which is claimed to depend on lowered capacity of the kidney to deaminate AL and GL (Russel and Wilhelmi, 142) and to form carbohydrate from these amino acids and other members of the Krebs cycle (Russel and Wilhelmi, 143; cf. 144). Treatment with adrenal cortical extract or desoxycorticosterone restores the rate of deamination to normal levels. (*l*-Amino acid oxidation is not impaired in the livers of adrenalectomized animals, 77, 101).

The increase of protein catabolism in hyperfunction of the thyroid (Graves' disease, fever) is likewise associated with excessive breakdown of carbohydrate and can be counteracted by large dietary allowance of carbohydrate. Tsitovskaya (154) observed a marked increase of amino nitrogen formation from NH<sub>3</sub>+PU in liver slices from thyroxine-treated animals. In the light of the data of Kritzmann and Melik-Sarkissyan, this finding points to the involvement, in the metabolic effects of thyroxine, of reactions relating to the dicarboxylic acid system.

The increase of protein metabolism associated with deficiency or deranged oxidation of carbohydrate, the effect of restored availability of carbohydrate in sparing protein or promoting its regeneration, and the regulatory functions of the dicarboxylic acid system in correlating protein and carbohydrate metabolism in the animal body, bear a remarkable resemblance to the analogous relations in the plant. The following passage from Chib-

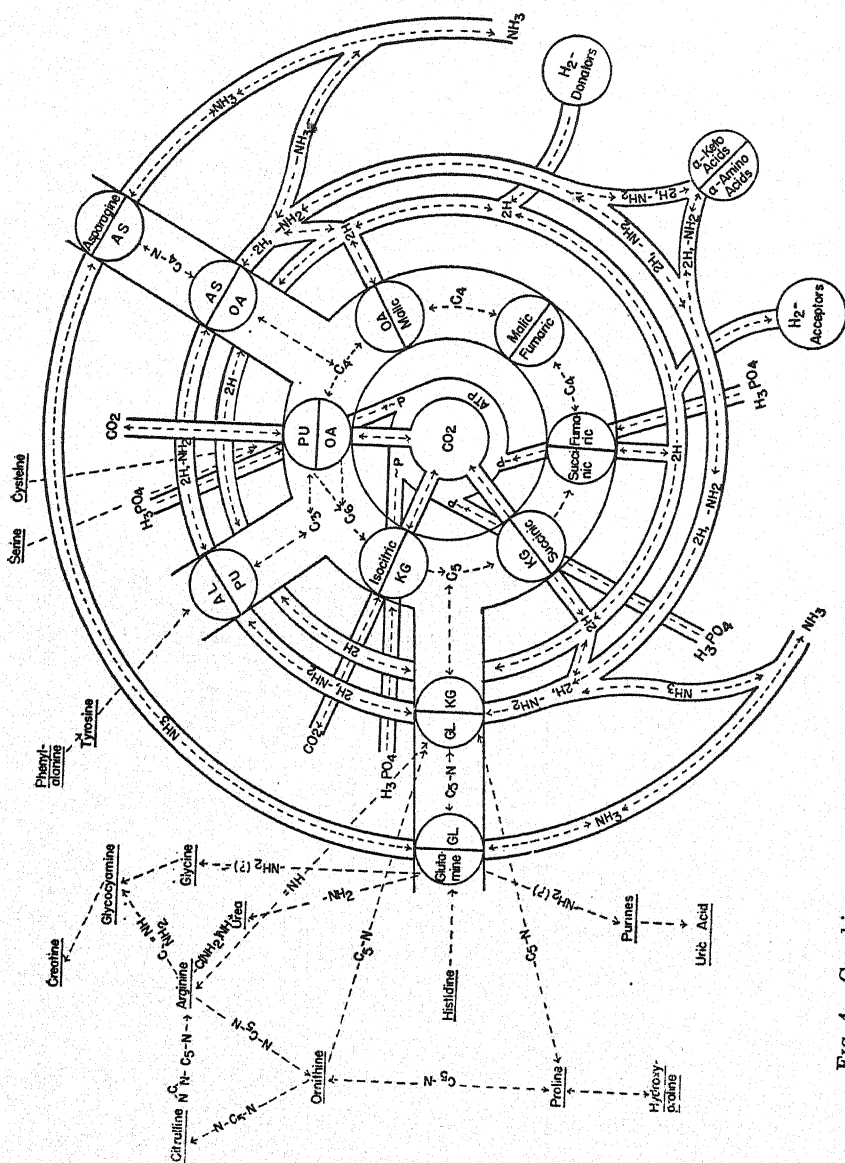


Fig. 4. Graphic summary of the principal metabolic functions of the dicarboxylic acid system.

nall's book on protein metabolism in the plant (55, p. 241) could be applied to animal metabolism almost unaltered and with equal justification:

"... my reading of a very extensive, if scattered literature, during the preparation of this book, has impressed me with a firm belief that it is the group of substances we refer to as "organic acids"<sup>1</sup> — which have, in the past, been neglected in studies of plant metabolism because satisfactory methods of characterization were not available — that occupy the central, and therefore the key position, in the carbohydrate, protein and fat metabolism of plant cells."

### CONCLUSION

In the foregoing pages a general picture has been given of the scope and significance of the manifold interrelations between the dicarboxylic acid system and various aspects of the metabolism of nitrogenous and non-nitrogenous body constituents. The more important interrelations are graphically summarized in Fig. 4. The central position of the dicarboxylic acid system in tissue metabolism is clearly demonstrated by the role of its components in respiratory hydrogen transport, in aerobic phosphorylation, in the formation, interconversion and breakdown of amino acids, in the fixation and liberation of carbon dioxide and of ammonia, in the synthesis of specific nitrogenous cell substances and end products. The interplay of these processes and their reciprocal control is largely based on the collector, shunting and distributive functions of the dicarboxylic acid system, offering numerous points of application for the regulatory influence of humoral agents — hormones, enzyme activators and inhibitors, ions,  $O_2$ ,  $CO_2$ ,  $NH_3$ , *etc.*, — and of neural factors.

The designation of this system as a central component of first order in the chemical integration of nitrogenous — and not only nitrogenous — metabolism is therefore fully justified.

Herewith we get the clue to the physiological significance of transamination. The role of transamination is evidently not confined to its participation — whether of restricted or of broader scope — in the synthesis, breakdown and transfer of amino groups, nor to the regulation of cellular oxidations by the formation and removal of respiratory mediators. Transamination provides for rapid interconversion of six major specific constituents of the dicarboxylic acid system — GL, AS, AL, KG, OA and PU. Accordingly, the aminopherases hold an eminent position in the chemical integration of nitrogen metabolism, on a par with aminodicarboxylic acid dehydrogenases, amidases and the enzymes of the Krebs cycle.

This conception satisfactorily accounts for the widespread occurrence of transamination and for its exceptionally high activity in certain tissues.

<sup>1</sup>*I.e.*, mainly di- and tricarboxylic acids (A. Braunstein).



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*Editors' note* (see p. 14) — The attention of the reader should be called to the important paper of Green, Leloir and Nocito (82a), concerning the purification of transaminating enzymes from pig heart, their specificity, and the role of pyridoxal phosphate as coenzyme. This paper was not available to Prof. Braunstein at the time when this review was written.

At the American Chemical Society meeting in Chicago (Sept. 1946), S. R. Ames and C. A. Elvehjem presented further evidence that pyridoxal phosphate acts as coenzyme for transamination in rat tissues; studies on the structure of pyridoxal phosphate were presented by I. C. Gunsalus and W. W. Umbreit, and by D. R. Heyl, S. A. Harris and K. Folkers. See also references 37a, 79b, and 128b.

The present note was written before the receipt of Dr. Braunstein's Addendum, which appears on the following pages and covers the same ground more fully.

## ADDENDUM IN PAGE PROOF

1. The  $\alpha$ -hydrogen of some other  $\alpha$ -amino acids which are not available for transamination with glutamic-alanine aminopherase (aspartic acid, leucine) or with any known transamination system (glycine) also undergoes dissociation upon incubation with active or boiled aph. With all amino acids excepting GL,  $\alpha$ -hydrogen labilization takes place only in the presence of an  $\alpha$ -keto acid, which can be replaced by glyoxylic acid, but not by acetone. With GL in the absence of ketoacid, the labilization of  $\alpha$ -hydrogen by aph is inhibited by phenylhydrazine and hydroxylamine. This indicates the participation of a catalytically active CO-group of aph in the hydrogen labilization reaction, but phosphopyridoxal (see below) is inactive in absence of the protein moiety of aph (Konikova, Dobbert and Braunstein, 175).

It appears that the dissociation of  $\alpha$ -hydrogen is an independent, relatively non-specific and heat-resistant function of aminopherase, prerequisite for but not necessarily followed by transfer of  $\text{NH}_2$ -groups (and electrons), i.e., by transamination proper. This enzyme-catalyzed exchange of stably bound hydrogen affords an explanation for the intravital replacement of  $\alpha$ -hydrogen in amino acids in isotope experiments (179, 172, 159, 19), usually interpreted as evidence for reversible deamination, resp. for dehydrogenation at the  $\alpha$ -carbon of the amino acids.

2. Green, Leloir and Nocito (173) have obtained from pig heart muscle highly purified preparations of two specific aminopherases — "glutamic-alanine transaminase" ( $\text{aph}_{\text{GL} \rightleftharpoons \text{AL}}$ ), identical with the enzyme of Lenard and Straub, and "glutamic-aspartic transaminase" ( $\text{aph}_{\text{GL} \rightleftharpoons \text{AS}}$ ), which has also been prepared by Schlenk and Fisher (177) by a different method.  $\text{Aph}_{\text{GL} \rightleftharpoons \text{AS}}$  is salted out from muscle extracts at higher concentrations of  $\text{NH}_4$ -sulfate than  $\text{aph}_{\text{GL} \rightleftharpoons \text{AL}}$ . The electrophoretic, ultracentrifugal and diffusion measurements of Green and associates (173) show that their  $\text{aph}_{\text{GL} \rightleftharpoons \text{AL}}$  preparation contained one active component (m.w. about 180,000); the  $\text{aph}_{\text{GL} \rightleftharpoons \text{AS}}$  preparation, while monodisperse in the ultracentrifuge (m.w. 60,000), consisted of two active fractions with different electrophoretic mobilities ( $2.9 \times 10^{-5}$  and  $4.9 \times 10^{-5}$ ). The "turnover number" of  $\text{aph}_{\text{GL} \rightleftharpoons \text{AL}}$  (8600 per minute) is about 13 times as high as the turnover number of  $\text{aph}_{\text{GL} \rightleftharpoons \text{AL}}$  (640 per minute), but the calculations of Green indicate that pig heart contains only about 0.12% of  $\text{aph}_{\text{GL} \rightleftharpoons \text{AL}}$  and as much as 1.6%  $\text{aph}_{\text{GL} \rightleftharpoons \text{AS}}$  (on a dry weight basis), which accounts for the superior rate of reaction  $\text{GL} \rightleftharpoons \text{AS}$  in the tissue.

The prosthetic group of both enzymes has been identified with phosphopyridoxal. Using an enzymatic assay with dopa-apodecarboxylase, Green and associates (173) found a codecarboxylase activity corresponding to a content of 0.55  $\mu\text{g}$ . phosphopyridoxal per mg. of maximally purified  $\text{aph}_{\text{GL} \rightleftharpoons \text{AS}}$  ( $Q=14,400$ ), and to 0.27  $\mu\text{g}$ . phosphopyridoxal per mg. of

aph<sub>GL $\rightleftharpoons$ AL</sub> (Q=64,000). The phosphopyridoxal equivalent of a Lenard-Straub preparation of aph<sub>GL $\rightleftharpoons$ AL</sub> (Q=18,000), made in the writer's laboratory, in the tyrosine apodecarboxylase test (Gale and Tomlinson) was 0.18  $\mu$  per mg. enzyme (171). Aph<sub>GL $\rightleftharpoons$ AS</sub> can be inactivated by visible or ultraviolet light, which destroys phosphopyridoxal (178). Lichstein, Gunsalus and Umbreit (176) have been able to restore the transamination activity of pyridoxine-deficient suspensions of *Str. faecalis* R and of an apo-aminopherase prepared from normal *Str. faecalis*, by the addition of phosphopyridoxal. Reversible splitting of aph<sub>GL $\rightleftharpoons$ AL</sub> of animal origin and its partial reactivation by boiled tissue extracts or synthetic phosphopyridoxal has been effected by Kritzmann and Samarina (174).

3. Purified aph<sub>GL $\rightleftharpoons$ AL</sub> or aph<sub>GL $\rightleftharpoons$ AS</sub> fail to catalyze the reaction AL $\rightleftharpoons$ AS. Green, Leloir and Nocito did not find an independent aspartic aminopherase in extracts from pig heart. They hold the opinion that reaction AL $\rightleftharpoons$ AS is the sum of reactions AL $\rightleftharpoons$ GL and GL $\rightleftharpoons$ AS, catalyzed by the two glutamic aminopherases with the participation of GL acting as an intermediary carrier (such a two-step transamination has in fact been described by the writer (24, 36; cf. p. 11, Table II). Green (173) supposes that aspartic aminopherase (116, 117) is a mixture of aph<sub>GL $\rightleftharpoons$ AL</sub> and aph<sub>GL $\rightleftharpoons$ AS</sub>, and that the activator of as-aph prepared by Braunstein and Kritzmann (37, "coenzyme of as-aph") is glutamic acid. This interpretation, while offering a satisfactory explanation of most experimental facts does not agree with the marked acid-lability of co-as-aph (37). Cross assays effected in the laboratories of Gale and Braunstein have shown that co-as-aph is not identical with phosphopyridoxal or with natural codecarboxylase, although concentrates of the latter contain some co-as-aph (171). A specific, non-dissociable as-aph is probably present in pigeon liver (131a).

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# Ferritin and Apoferritin

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## I. HISTORY

It may be said, without searching for specific quotations in the literature, that organic iron compounds, other than iron porphyrin compounds, had been surmised to exist in the organism for a long time. In 1894, Schmiedeberg (1) prepared an iron-containing protein from pig's liver, containing about 7% iron. This poorly defined compound was obtained in a denatured condition. In spite of several re-investigations by others no further progress in the matter was recorded for a long time. The first decisive step was made with Laufberger's discovery (2) of a readily crystallizable protein, containing as much as about 20% of iron, in the ferric state. It was obtained from horse spleen and other organs of different animals by means of a simple method which essentially depends on the fact that this iron protein readily crystallizes from its solution on addition of cadmium sulfate. In deference to Schmiedeberg's "ferratin," but in distinction from that questionable compound, Laufberger called his well defined, reproducible compound "ferritin." It has a surprisingly high iron content, even of a higher order of magnitude than that of hemoglobin, and even higher than that of the hemosiderin granula which were never considered as a definite chemical entity nor as a regular protein compound.

The next step in the clarification of the nature of ferritin was the observation, in the writer's laboratory (5), that the iron can be removed from ferritin, leaving a typical, colorless, iron-free protein which, on addition of cadmium sulfate, gives rise to crystals indistinguishable from



those of ferritin, except for their color. This protein proved to be different from all other proteins thus far known and was termed "apoferritin." Ferritin is brown, apoferritin is colorless. Apoferritin is a very homogenous material, easy to obtain in pure condition, having a molecular weight of 460,000, a typical protein, composed of amino acids, capable of denaturation by heat, containing no nucleoprotein nor any phosphorus, and resembling a globulin more than an albumin. The iron content of ferritin is not quite constant, around 20%, varying from 17 to 23%. This variability will be commented upon presently.

## II. PREPARATION OF FERRITIN

The richest source of ferritin is horse spleen, from which it may be prepared as follows. Mashed spleen is extracted with an equal amount of water for a day, filtered, the filtrate heated to 80°C. (not higher), and the coagulum separated by filtering through cheesecloth. To each 100 cc. of the red-brown filtrate is added 30 g. of ammonium sulfate. The precipitate is collected by centrifugation and dissolved in as small an amount of water as possible. After the addition of a solution of cadmium sulfate ( $\text{CdSO}_4 \cdot 8/3 \text{H}_2\text{O}$ ) so as to establish a final concentration of 5%, crystal-

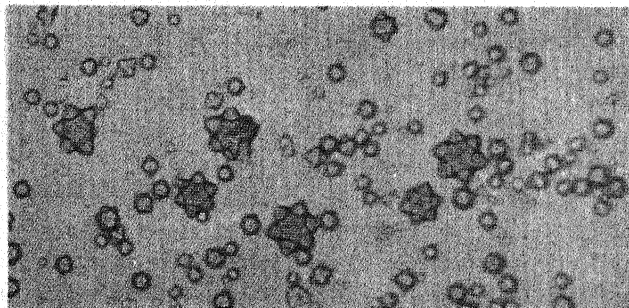


FIG. 1. Ferritin from horse spleen.

lization starts almost immediately. After standing overnight the brown crystals are separated by centrifugation from a brown mother liquor which no longer yields any crystals and will be referred to as "non-crystallizable ferritin." It may consist of some denatured ferritin and perhaps also of some other unidentified iron compounds. The crystals, when free from ammonium sulfate, are not readily soluble in water, but readily dissolve in 2% ammonium sulfate solution, yielding a deep red-brown solution from which they can be recrystallized by cadmium sulfate, over and over again, practically without loss. A protein impurity of

brown color, probably denatured ferritin, can be removed from a dialyzed solution of the crystals by bringing it to pH 4.6 with dilute acetate buffer (resulting in an ionic strength of  $< 0.1$ ). An amorphous brown precipitate arises and can be removed by centrifugation, and the now pure ferritin can be recrystallized with  $\text{CdSO}_4$ . This impurity may be present either in a negligibly small amount or sometimes to a higher extent. Crystals of ferritin can be obtained directly by mixing crushed tissue or tissue juice with 10% cadmium sulfate solution under a cover slide. Crystallization can be watched under the microscope.

### III. PREPARATION OF APOFERRITIN

The first step in removing the iron from ferritin consists in reducing the iron from the ferric to the ferrous state. Although neither Laufberger nor Kuhn succeeded in bringing about this reduction, it can be easily accomplished by addition of sodium dithionite ("sodium hydrosulfite,"  $\text{Na}_2\text{S}_2\text{O}_4$ ) to a slightly acidified solution of ferritin. At neutrality, reduction does not proceed at all, but becomes more rapid with increasing

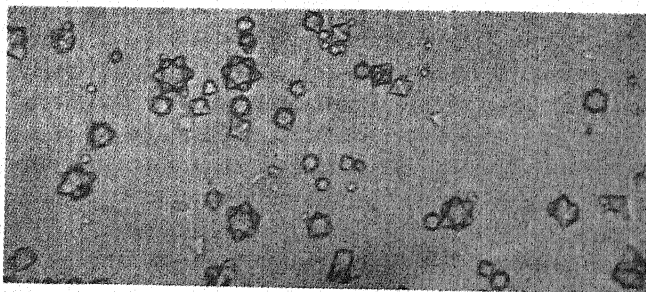


FIG. 2. Apoferritin from horse spleen.

acidification. A pH of about 4.6 provides the best condition and does not bring about any denaturation of the protein. The various methods tested in the laboratory differ only in slight details and amount to the same in principle. A solution of ferritin, brought to pH 4.6 by acetate buffer, is mixed with sodium dithionite and  $\alpha, \alpha$ -bipyridine in a cellophane bag which is entirely closed to prevent undue access of air. After standing overnight it is dialyzed against water. The red, soluble, ferrous complex of bipyridine dialyzes out, and the remaining solution is deprived of much of its iron. After addition of cadmium sulfate, crystals are obtained of much paler color than originally. The whole process is repeated until eventually the mother liquor yields, on addition of cadmium sulfate, perfectly colorless crystals. They are practically free

from iron. Once in a while the crystals may have a very faint yellowish-green tint, which is not due to iron but probably to cadmium sulfide; usually they are quite colorless. Apoferritin gives all the usual color tests for proteins; its hydrolyzate contains amino acids, of which several have been identified. The ultraviolet absorption spectrum suggests the presence of tryptophan and tyrosine, and according to an analysis by R. M. Archibald (personal communication) it contains 8% arginine. The absorption spectrum shows no evidence of any band characteristic of nucleic acid.

#### IV. PHYSICAL PROPERTIES OF FERRITIN AND APOFERRITIN

Apoferritin can be easily purified by recrystallization so as to represent a perfectly homogeneous material, with a sharp sedimentation boundary and a sedimentation constant  $S^{\circ}_{20} = 19.5$  Svedberg units, corresponding to a molecular weight of 560,000 for a preparation from horse spleen, and an asymmetry coefficient of 1.14, corresponding to axis ratio of about 1:3, *i.e.*, of the same order as egg albumin, according to Rothen (10). It is homogeneous also on electrophoresis. Ferritin, however, exhibits a diffuse sedimentation boundary, the brown color decreasing from the bottom to the top of the vessel. About 20% of the material on top is colorless and iron-free, representing apoferritin. The remainder obviously consists of molecular aggregates of the protein, the micelles being held together by ferric hydroxide, which probably forms micelles as in a colloidal solution of ferric hydroxide, which in their turn, are combined with the protein, linking two or more protein molecules to an aggregate of larger size. This inhomogeneity is not found on electrophoresis. Here a sharp boundary is established. These two findings are not irreconcilable, since the rate of sedimentation depends on molecular size, but the rate of electrophoresis on the number of electric charges per unit surface of the elementary particle independent of its size. The sharpness of the sedimentation boundary and the high stability of apoferritin have made this protein a suitable material for some fundamental investigations of the ultracentrifuge method. It was utilized in this respect by Rothen (10).

Ferritin, in solution, coagulates on heating. When the temperature does not exceed 80° (this temperature threshold depending somewhat on the concentration), a precipitation occurs which, according to concentration, appears either as a turbidity, or as a voluminous, heavy precipitate. On cooling down to room temperature, the whole of the precipitate is redissolved to give a clear solution (4). After heating to somewhat higher temperatures, the reversal of the coagulation at low temperature is sluggish and incomplete, and after heating to 100°C. the coagulation is entirely irreversible on cooling. In contrast herewith,

apoferritin coagulates at higher temperatures always in an irreversible manner. The coagulum of ferritin is brown, that of apoferritin is colorless.

The crystal form of all ferritin or apoferritin preparations from any animal species from which they can be obtained, are of the cubic system and optically isotropic. Usually they are octahedra and, especially in horse ferritin and apoferritin, twinned forms, or rather tripled forms occur. These almost give the impression of a cube with a tetragonal pyramid upon each of its six faces. The same shape has been found by Kuhn *et al.* (3) for ferritin from the jackal. Preparations from other animals usually show either simple octahedra, sometimes also cubes, or tetrahedra. These crystals are always very regularly built, and may, under proper conditions, readily grow to the size of 1/10 of a millimeter and even much more. Only human ferritin and apoferritin has a less regular shape, with slightly curved faces, which may be just visualized as imperfect octahedra. In all cases, good crystallization can be accomplished only on addition of a large excess of cadmium sulfate. Using concentrations of  $\text{CdSO}_4$ , below the optimal one, sometimes the transient formation of crystals in the form of thin, anisotropic plates was observed. With still lower concentrations of  $\text{CdSO}_4$  an amorphous precipitate arises, which on addition of more  $\text{CdSO}_4$  gives rise to regular crystallization. Zinc sulfate seems to have, in principle, the same effect as cadmium sulfate, but it is not as reliable as cadmium sulfate. The difference in behavior of insulin and ferritin is of interest. Insulin crystallizes in the presence of a trace of zinc salt and needs no excess of it beyond what is obviously incorporated in the crystal lattice. Ferritin or apoferritin crystallizes only in the presence of very much more of the metal salt than what can be imagined to be incorporated in the lattice. The excess obviously stays in the interstices of the lattice. It can be removed, in part, by washing the crystals with 10% KCl solution, but the Cd content does not fall below 1.6%. The content of water, or better, of mother liquor, of the crystals in apoferritin, may be estimated as about 45%. Considering the similarity of the crystal lattice of ferritin and of apoferritin, to be discussed presently in greater detail, one must infer that a great deal of the water which is enclosed in an apoferritin crystal, is replaced, in a ferritin crystal, by ferric hydroxide in such a way as not to interfere with the crystal lattice of the protein. These observations render it rather difficult to interpret in which form the iron is embedded in ferritin. However, the observations of ultracentrifugation, on crystallization, and on the X-ray diffraction pattern presently to be discussed, leave no alternative to the suggestion that the iron is present in the form of colloidal micelles of ferric hydroxide, attached by some unaccountable

affinity to the protein. The idea that ferritin might be simply a ferric salt, or a ferric complex compound of the protein, with a well-defined stoichiometrical ratio of iron to protein, each iron atom being attached to some side-chain of the protein, is not reconcilable with the observations. Kuhn has pointed out that the iron content is such that the ratio of iron atoms to CONH groups is approximately 1:1. Even if this statement should be corroborated, it may be no more than a coincidence. There are too many evidences against the hypothesis that the iron be "atomically dispersed" in ferritin. The iron atoms seem to be dispersed in clusters, rather than as single atoms.

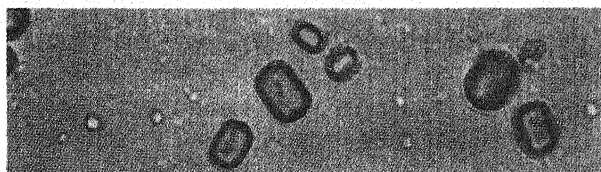


FIG. 3. Ferritin from guinea pig liver.

The slight phosphorus content of ferritin is not a constituent of its protein, since apoferritin is free from phosphorus. It should rather be considered as combined with the iron of ferritin. The iron compound in ferritin may be imagined (10) to be of the composition, approximately,  $(\text{FeOOH})_8(\text{FeO}:\text{OPO}_3\text{H}_2)$ , or one phosphate group for about nine iron atoms.

A solution of ferritin mixed with sodium hydroxide gives rise to a brown precipitate of ferric hydroxide, which is not precisely of the composition  $\text{FeOOH}$ , but contains some phosphate, just as all amorphous ferric hydroxides, especially those obtained from coagulation of any colloidal ferric hydroxide sol, contains some anionic constituents other than  $\text{OH}$ . The phosphorus content of that precipitate is smaller than would correspond to the ratio 1:9 mentioned above. A part of the phosphate seems to be split off from its attachment to iron, when the iron is precipitated by sodium hydroxide.

X-ray diffraction patterns have been worked out by Fankuchen (8) with the Debye-Scherrer powder method, which, because of the cubic nature of the crystals were quite helpful in some respects for the interpretation of the structure. Fankuchen obtained patterns reconcilable with a face-centered cubic unit cell, and the pattern was the same both in ferritin and in apoferritin. The distances of the lines were the same, but there was a difference in the intensities of some lines, those of ferritin being more intense than those of apoferritin. This seems to show, once

more, that the pattern of the crystal structure is brought about by the protein and is not essentially changed by the addition of iron. Crystals big enough for the single-crystal method are available now and wait for re-investigation.

#### V. ATTEMPTS OF SYNTHESIS OF FERRITIN FROM APOFERRITIN

Although the iron can be removed from ferritin without difficulty, no unobjectionable procedure has been found as yet to achieve the synthesis of ferritin from apoferritin and any iron compound. When a solution of apoferritin is mixed with any one of many iron salts, either in the ferrous or the ferric state, coagulation and denaturation takes place. When the iron is added to the crystals of apoferritin in the form of any of the manifold colloidal solutions of iron hydroxide, nothing happens. The iron does not enter into the crystal lattice. This is quite in contrast with what happens when a diluted solution of crystal violet is added to the crystals of apoferritin. Then the crystal stains deep-violet throughout, not only at the surface, showing not only penetration but even accumulation of the dye within the crystal. Very likely the micelles of any of those colloidal ferric hydroxide sols, which can be prepared in the laboratory, even though possibly highly dispersed, are too big to enter the crystal lattice of apoferritin.

Only under one condition can something resembling a synthesis be accomplished (4). It has been mentioned that after the completion of crystallization of ferritin, there remains a brown mother liquor from which no further crystals can be obtained. When apoferritin is dissolved in this brown mother liquor and  $\text{CdSO}_4$  added, brown crystals of ferritin are formed.

Later studies by Granick and Hahn (11), although not conveying a clear-cut picture, seem to suggest that the synthesis of ferritin from iron and apoferritin, is due to a specific, probably enzymatic process.

#### VI. MAGNETIC PROPERTIES OF FERRITIN

The iron of ferritin is in the ferric state, is paramagnetic, and has a magnetic susceptibility per gram-atom of Fe, which is constant and reproducible, and characteristic of the ferritin iron (6). So far no other physiological iron compound has been found to have the same susceptibility. The peculiarity of this behavior will be best understood by some general remarks on the magnetic susceptibility of ferric compounds.

Ferric ion,  $\text{Fe}^{+++}$ , has an incomplete 3d sub-shell of electrons. This sub-shell, when fully occupied, would contain ten electrons. In  $\text{Fe}^{+++}$  there are only 5. There are three conditions in which those five electrons may exist: either all with parallel spins, or all paired as much as possible

so that only one electron remains unpaired, whereas the remaining four form two pairs, each with electrons of opposite spins; the third possibility is that only two of the five electrons are paired and there remain three electrons with parallel spins. If the paramagnetic susceptibility should depend only on the electron spin, and orbital contributions of the electrons can be neglected, then according to quantum mechanics, the magnetic dipole moment,  $\mu$ , expressed in Bohr magnetons, depends upon the number,  $n$ , of unpaired electrons, as follows:

$$\mu = \sqrt{n(n+2)}$$

Supposing the validity of Curie's law (which within certain limits of temperature has been approximately verified for ferritin) the magnetic susceptibility,  $\chi$ , per gram-atom of iron, should be related to  $\mu$  as follows:

$$\mu = 2.84\sqrt{\chi T}$$

where  $T$  is the absolute temperature.

For 22°C. the following values of  $\chi$  are expected from the theory, according to the number of unpaired electrons,  $n$ :

$n =$	1	3	5
$10^6 \chi =$	1,260	6,300	14,700

Furthermore, quantum theory requires that for  $n=5$  (or, for that matter, also for  $n=10$ ), that is, for a completely filled 3d sub-shell, or for a half filled 3d sub-shell, there are no orbital contributions to the magnetic moment. In all other cases small orbital contributions may be expected, which cannot be predicted with accuracy but may sometimes be as high as  $\frac{1}{2}$  Bohr magneton. So, for ferric chloride (in an acid solution to prevent hydrolysis), at room temperature,  $\chi$  is within the limits of error,  $= 14,700 \times 10^{-6}$ , whereas on the other hand,  $\chi$  for ferricyanide ion is 2,300, instead of the expected 1,260; or, the moment,  $\mu$ , is 2.3 instead of the expected 1.73 Bohr magnetons. In ferritin  $\chi \times 10^6$  is found to be  $6,145 \pm 150$  at 22°C., and approximately 6,700 at 2°C., which is within the limits of error just the value for three unpaired electrons without any orbital contribution. So it is most probable that the iron in ferritin has three unpaired electrons. Taking this result for granted, it is noteworthy, but not contrary to theory, that no measurable orbital contributions to the magnetic susceptibility can be detected.

The constancy and reproducibility of the magnetic susceptibility of the iron atom in ferritin is even more remarkable as the susceptibility of artificially prepared colloidal ferric hydroxide was found to be very variable, according to the method of its preparation. Although it is



constant in time for any individual ferric hydroxide sol (unless exposed to very high temperatures on long refluxing),  $10^6 \chi$  may vary from 14,000 down to 2,700 (or  $\mu$  from 5.9 to 2.5 B.M.), with all intermediate values occurring. Obviously there is a mixture of iron atoms in different magnetic states. The ferric hydroxide of ferritin, however, seems to be

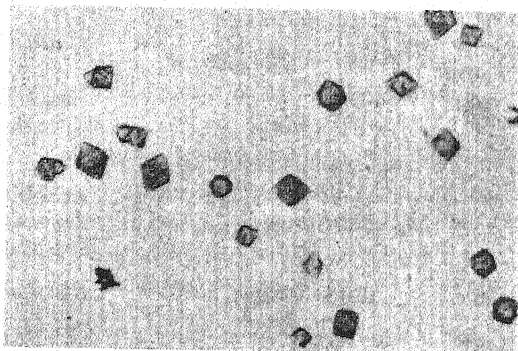


FIG. 4. Ferritin from mouse liver.

homogeneous with respect to the magnetic state of the iron. This statement cannot be proved with certainty since a certain mixture of iron atoms in different magnetic states may, by coincidence, bring about the characteristic susceptibility of ferritin iron. At any rate, disregarding the proposed interpretation, the susceptibility of ferritin iron is constant and specific, and different from all other iron compounds physiologically occurring. Even the iron of the ferric hydroxide precipitated from ferritin by sodium hydroxide has the same magnetic susceptibility, within the limits of error.

The only compound known so far in which ferric iron is magnetically comparable with that of ferritin is methemoglobin (ferri-hemoglobin) in alkaline solution, which is the first case in which this particular level of susceptibility has been discovered, in work done by Pauling and Coryell (12). Here the susceptibility is slightly higher, suggesting some orbital contribution to the susceptibility.

#### VII. OCCURRENCE OF FERRITIN AND APOFERRITIN

Ferritin was first discovered by Laufberger in horse's spleen, and to a smaller extent in liver. Kuhn, Sørensen and Birkofer (3), in 1940, obtained ferritin also from the spleen of dog, cat, jackal, but not from the spleen of guinea pig, or rabbit. However, Granick (7) obtained it also from the two latter animal species. The following table, according



to Granick, summarizes the occurrence of ferritin in various animals and organs. Its abundance is indicated by the number of + marks, the numbers in parentheses represent the extremes of ferritin found in many individual cases, expressed in terms of grams per 100 g. of fresh weight of organs.

<i>Species</i>	<i>Spleen</i>	<i>Liver</i>	<i>Red bone marrow</i>	<i>Other Organs</i>
Horse	++++ (0.10-0.01)	+	++++	Testes + Kidney, pancreas, brain, stomach mucosa, striated muscle, blood; all 0
Human	++	+++ (0.09-0.0024)	++	Kidney + Pancreas, pituitary, ovary: 0
Dog	+	(0.015-0.000) +	0	All other organs: 0
Mouse	+	++		Testes + All other organs: 0
Rat	+	+		Kidney: 0
Pig	+	+		Testes + Kidney: 0
Rabbit	+	0	0	Testes +
Cat	0	0		Kidney +

No ferritin was found in tissues of cattle, sheep, deer, chickens, or bull-frog. The ferritin prepared from testes of horse, mouse, and rabbit was of paler color than usual, indicating a lower iron content. Later investigations by Granick, not yet published, supplement this table with respect to the ferritin content of the intestinal mucosa of the guinea pig. It may be just mentioned preliminarily here, that under conditions of normal feeding small amounts of ferritin can be found in the intestinal mucosa from the duodenum down at least to the ileum; that the ferritin content is very markedly increased on feeding with ferrous ammonium sulfate, beginning from about five hours after the intake of the iron salt and lasting for several days. It is important to state that apoferritin was never found in the intestinal tract, nor in the liver, spleen or bone marrow. It would be a mistake to assume that apoferritin is present all the time and according to the iron intake is converted into ferritin. Rather does the protein apoferritin seem to be synthesized at the moment when it is needed for storage of iron and to disappear as its iron is released for the formation of hemoglobin. It is noteworthy that ferritin or apoferritin was never found in blood or in muscle, not even by the

very sensitive precipitin reaction presently to be discussed. So the alluring idea that iron might be transported through the bloodstream, in the form of ferritin, must be discarded. The iron which is always found in the hemoglobin-free blood plasma is not in the form of ferritin. It is probably carried along with the serum proteins.

### VIII. IMMUNOLOGICAL PROPERTIES

Ferritin or apoferritin, several times recrystallized, injected into rabbits subcutaneously, behaves as an antigen and readily produces a specific antibody in the form of a precipitin (7). Ferritin and apoferritin are indistinguishable immunologically. The precipitin is specific for the animal species but not for the particular organ from which it is prepared. This precipitin test is very sensitive and reveals the presence of ferritin in such organs as are too low in ferritin content to allow ready

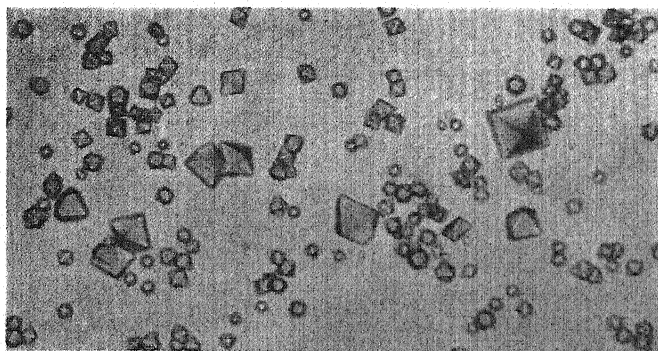


FIG. 5. Ferritin from dog liver.

preparation of crystals. Even the brown mother liquor, after crystallization, which yields no further crystals, gives a positive precipitin reaction with the antiferritin serum. It is mainly on the basis of this immunological test that the absence of ferritin in blood and in muscle can be asserted with certainty. As regards the degree of specificity, dog apoferritin gave a weak precipitin reaction with the antiserum to horse spleen apoferritin, but human apoferritin gave no reaction with antihorse serum. So, slight overlapping of the immunological species specificity may occur, but in general, species specificity is established beyond doubt. This is also in accord with the fact that the crystals obtained from different animal species are quite readily distinguishable.

## IX. METABOLISM OF FERRITIN

As may be surmised from its chemical composition, ferritin is used as a source of iron to be used for hemoglobin formation and to store liberated iron ensuing from the destruction of hemoglobin. Evidence for such an assumption can be found in the following experiments carried out with radioactive iron (9).

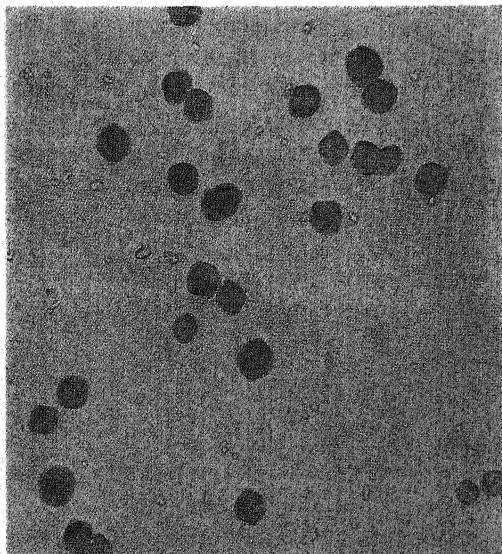


FIG. 6. Ferritin from human liver.

When a dog is made anemic, then injected with radioactive ferric ammonium citrate, after a few days about 80% of the radioactivity was recovered in the form of ferritin (crystallized and "non-crystallized" together), prepared from the liver. Very little was recovered from the spleen. On the other hand, when a dog was made anemic and its blood replenished with radioactive hemoglobin contained in red blood cells, which was previously prepared in another dog, the radioactivity was, to a large extent, recovered from ferritin in the crystalline state prepared from both liver and spleen. So, if it be legitimate to generalize from a few experiments, inorganic iron in the dog is stored up mainly as liver ferritin, whereas iron derived from hemoglobin decay is stored up as ferritin in both liver and spleen. Furthermore, the spleen of a horse used for a long time for manufacturing diphtheria serum and killed in a state of anemia, contained a quite unusually small amount of ferritin.

Another horse, after complete recovery from anemia, yielded the customary amount of ferritin from its spleen.

There is a very striking feature with respect to ferritin production in the organism which should be especially emphasized. It has been mentioned that the iron-free protein apoferritin can be readily prepared by a chemical procedure from ferritin. Furthermore, small amounts of apoferritin, or at least a ferritin of low iron content, can be prepared from some organs not involved in the formation of hemoglobin, especially from testicles and kidneys. However organs involved with the formation or destruction of hemoglobin, *viz.*, spleen, liver, bone marrow, never yielded any ferritin of low iron content (say below 17% Fe). From such organs ferritin can be obtained but never even in the most anemic condition any compound very low in iron content and resembling apoferritin. One might have expected apoferritin to be present always, and according to the need to absorb iron so as to form ferritin. This does not seem to be the case. The protein apoferritin appears to be synthesized at the moment when it is to be used for the storage of iron. Another experimental procedure soon to be published by Granick conforms with this point of view. In a normal guinea pig very little, if any, ferritin can be found in the intestinal mucosa. On feeding iron as ferrous ammonium sulfate, a few hours after the feeding the intestinal mucosa is abundant with ferritin, which can be seen as brown octahedra when the mucosa is mixed with cadmium sulfate solution under a cover slide. This condition seems to remain until about five days after the feeding with iron. Without feeding iron, only very few crystals of ferritin and never any of apoferritin can be found. So the presence of iron appears to be the stimulus for the synthesis of apoferritin which stores the iron in the form of ferritin, and that specific protein disappears again when its iron is released for the formation of hemoglobin (or other iron-porphyrin compounds). The significance of those small amounts of low iron content, in testicle, etc., remains unexplained. Typical ferritin is found to an appreciable extent in all organs connected either with the formation or the destruction of hemoglobin; spleen, liver, red bone marrow, and with those organs which physiologically absorb the iron of the food, namely, the intestinal mucosa.

When a new kind of protein is discovered, there is always the suspicion that it may be an enzyme or coenzyme. So far no chemical process, either of specific hydrolytic or oxidative-reductive character, has been found for which apoferritin would function either as an enzyme or as a coenzyme. Its sole function appears to be storage of iron. Why just this particular protein, apoferritin, is able to store iron is not understood.

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# Adsorption Analysis of Amino Acid Mixtures

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## I. INTRODUCTION

No doubt the common procedures for analyzing protein hydrolyzates for amino acids despite some notable advances during the last years need improvement. Too much material is required and too much time consumed if more than a few of the most important amino acids are to be determined. There are a number of more or less specific micro methods available, but during the last years attention has been directed to two methods of more general usefulness both for the separation and determination of small quantities of amino acids, namely the adsorption and the microbiological methods. The present review will deal only with the former and does not aim at completeness (which would meet with great difficulties as all literature on the subject is as yet not available to the author) but is intended mainly to emphasize some aspects of the problem which have played a role in the work in the author's laboratory.<sup>1</sup>

It is natural that the great success in the application of the chromatographic method to various separation problems in organic and biochem-

<sup>1</sup>At the time when this review was written, the paper by Martin and Synge in Volume 2 of "Advances in Protein Chemistry" was not available to the author. This has led to some overlapping between the subjects discussed in their article and in the present contribution. However, this overlapping is not serious and it has seemed desirable in every way to give in full the present, more detailed discussion of the methods developed in the Uppsala Laboratories. (*Editor's note.*)

istry should inspire attempts to apply similar procedures for amino acids and protein breakdown products in general. On the other hand chromatography in aqueous solutions has not yet been worked out so well as in organic solvents and in many cases presents some difficulties mainly because there is no such great variety of elution agents available. The fact that substances are uncolored adds to the difficulties in the case of amino acids and peptides. The advantages of the method, namely great specificity, easy handling of small quantities and rapidity should, however, be of particularly great value in this field. Moreover, it should be possible in many cases to extend the method from the amino acids to peptides and protein split products in general.

Much work has been done in the field during the last few years mainly in England, the United States, Germany, Czechoslovakia and Sweden, and several alternative methods have been tried with varying degrees of success. The present status of the problem seems somewhat confused—no doubt mainly depending upon the difficulties in communications between different laboratories during the war. It seems definite, however, that adsorption methods will offer possibilities for separation of most amino acids quantitatively on a semi-micro scale but there is considerable uncertainty about the accuracy attainable and about the selection (and combination) between the various possible modifications of the procedure which have been tried.

For a general orientation in the field of adsorption analysis the reader is referred to the monographs by Zechmeister and Chohnoky, by Strain (1942) and by Hesse (1943). The application to amino acid analysis has been reviewed by Th. Wieland (1943) but a general discussion of the subject is also found in several of the papers quoted below.

## II. SOME REMARKS ON THE PRINCIPLE UNDERLYING THE METHOD

The ideal case of adsorption separation may be realized with an adsorbent combining strongly only with the substance to be separated out of a mixture and showing no or a very weak affinity for the other components. Such cases may often be realized by suitable choice of adsorbent and solvent in the case of substances soluble in organic solvents. Thus in one single operation a substance or a group of substances may be separated out of a mixture. A good illustration of this type of work is given by Trappe (1940, 1941) who worked out a scheme for fractionation of lipid mixtures in successive groups by stepwise variation of adsorbent and solvent. Also some of the work with amino acids quoted below follows similar lines. In such cases no special apparatus is needed, the completeness of the separation can be controlled with suitable reagents in the filtrate. In a great many cases however the differences in adsorption

affinity are not sufficiently large to allow a separation of the "all or none" type. Then one makes use of the differences in affinity for attaining a separation by differences in the rate of migration of zones of the substances in a chromatographic column (the Tswett chromatographic technique). Theoretically this method should be capable of separating substances with very small differences in adsorption capacity. The rate of migration of a zone depends upon the adsorption coefficient, as has been shown by several authors (see for example J. Norton Wilson, 1940; Martin and Synge, 1940; and Tiselius, 1940, 1943).

Suppose  $w$  is the volume rate of migration for a zone in ml. per sec and  $V$  the volume of the solution pressed into the column in ml. per sec.,  $s$  the specific gravity of the (dry) column,  $\Delta$  the volume of solvent enclosed in the column per gram of adsorbent during the process, and  $\alpha$  the adsorption coefficient of the solute at the concentration in question, that is the ratio between the amount  $m$  adsorbed per gram of dry column material and the concentration  $c$  in the solution. One obtains easily

$$\frac{w}{V} = \frac{1}{s(\alpha + \Delta)} \quad (1)$$

that is, the rate of migration of a zone is very nearly inversely proportional to the adsorption coefficient for the material in that zone at the concentration in question. Two substances with different  $\alpha$  should therefore always be completely separable if only a sufficiently long column were available. For everybody who has worked with chromatography it is well known that this is far from being the case, and that only very marked differences in adsorption will allow a complete separation in this manner, for several reasons. First of all the adsorption isotherm is almost never a straight line, and so  $\alpha$  increases with decreasing concentration. This tends to make the front of a zone quite sharp but makes the tail gradually more diffuse as the more slowly migrating parts of the zones where the concentration is lower lag behind ("tailing" of a zone). However, there may be other causes of tailing. The equation above is deduced under the assumption that adsorption equilibrium is established rapidly as compared with the rate of flow  $V$  of the solution. Direct determinations of rates of adsorption, however, show that this is not always the case and that attainment of equilibrium may require hours or days. If that is the case marked tailing will occur unless  $V$  is very small. Still another source of disturbance should be mentioned. It seems to be very difficult to pack a column of adsorbent so evenly that the zones are not more or less distorted when migrating. The author and his collaborators have devoted much attention to this difficulty and tried various methods of making the packing procedure less dependent upon chance,



as yet however without much success.<sup>1</sup> By far the best results are obtained with active carbon. This kind of disturbance is perhaps less serious when the column is finally cut into pieces and the various zones extracted with suitable solvents than when the zones are allowed to migrate down into the filtrate, as is mostly the case in amino acid analysis.

The two first-mentioned sources of disturbance, however, entirely depend upon the nature of the adsorbent, and possibly they may in the future be eliminated to a large extent if by systematic investigation more suitable adsorbents are found or can be prepared. So far one has had a rather limited choice of adsorbents which have been prepared without much regard to the use for the purpose in question. The new synthetic organic permutites (see p. 88) have opened up a new field, and now it is evidently possible to regulate the electrochemical properties of such adsorbents at will. Much remains to be done, however, to obtain constant adsorption coefficients and rapidly working materials. The first of these goals seem to be realized to a certain extent in the special kind of adsorbents used in "partition chromatography."

The above lines are just intended to emphasize, before the various methods of analysis are described, that the success of any of these procedures ultimately depends upon the properties of the adsorbent under the particular working conditions.

### III. ADSORPTION ANALYSIS BY SUCCESSIVE DETERMINATION OF THE CONCENTRATION OF THE FILTRATE

The methods worked out in the author's laboratory make possible a quantitative control of the adsorption analysis by continuous determinations of the concentration in the filtrate leaving the column, using optical methods depending upon the refractive index. A description of an early phase of this work is found in the author's paper in *Advances in Colloid Science* Vol. I, pp. 81-98 (1942). It was mentioned in this paper (p. 97) that a micro-interferometric arrangement for the observation was being constructed. This was completed and described shortly afterwards (Tiselius and Claesson, 1942) and further improvements have been made along the same lines (Claesson 1944, 1946). These methods are much superior to those described in the 1942 review and are now used exclusively. Similar work has been performed by Dutton (1944), also using refractive index measurements. Moreover, Cassidy and Wood (1941) and Cleaver, Hardy and Cassidy (1945) by analyzing successive portions of the filtrate also were able to follow the process in the chromatographic column.

<sup>1</sup> Vibration of the column during packing is often of advantage. An ordinary barber's vibration massage apparatus is useful for this purpose.

These methods are of practical importance mainly when closely similar substances have to be separated and therefore the "all or none method" is insufficient. Some of the results gained, however, are of a rather general interest for the understanding of the processes underlying adsorption analysis and therefore will be dealt with here, before a survey of the other methods of applying adsorption analysis to amino acids are discussed.

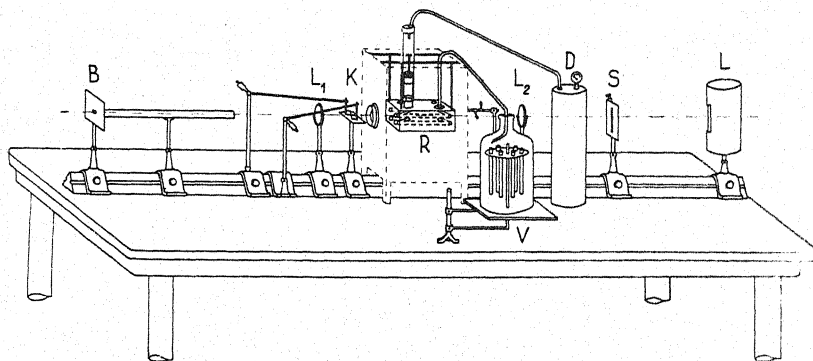


FIG. 1. Diagram of the microinterferometric arrangement for adsorption analysis (Tiselius and Claesson).

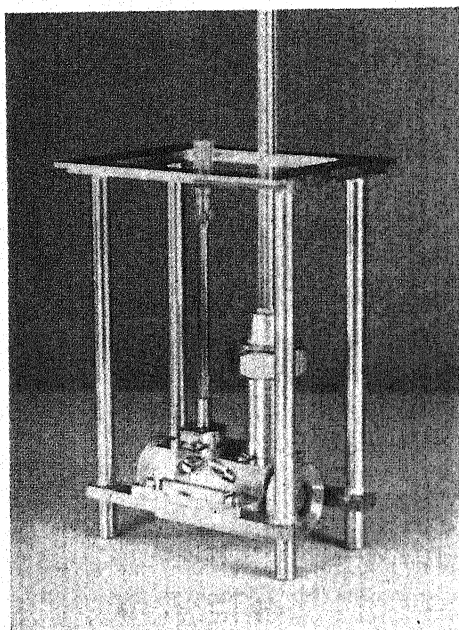


FIG. 2. Microinterferometer cell with burette and outlet.

The micro-interferometric arrangement of Tiselius and Claesson is shown diagrammatically in Fig. 1, where  $R$  is the interferometer cell where the solution flows from the adsorption cell through a gold capillary

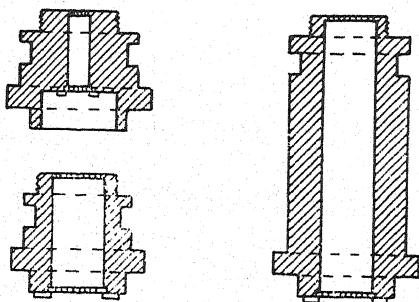


FIG. 3. Adsorption cells of volume  $1250\pi$ ,  $500\pi$ , and  $50\pi$  mm<sup>3</sup>.

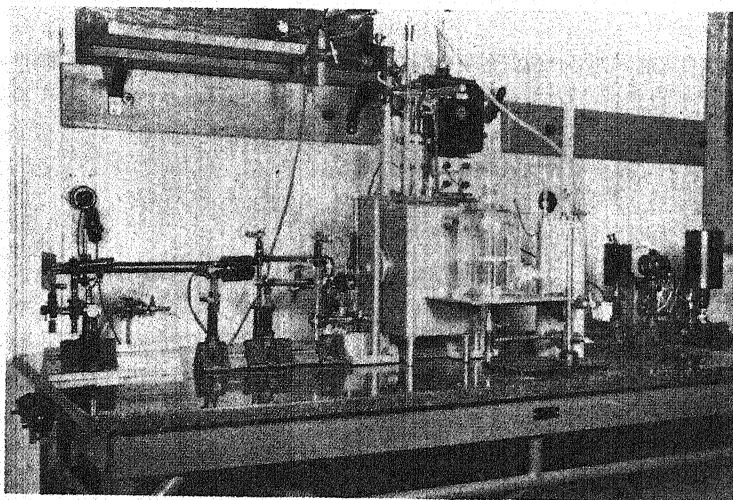


FIG. 4. Photograph of the arrangement for adsorption analysis by interferometric observation.

of 100 mm. length and 1.4 mm. diameter, and  $B, L_1, K, L_2, S$  and  $L$  the optical arrangement,  $D$  a compressed air cylinder,  $V$  a set of tubes for collecting samples of the solution leaving the interferometer. A photograph of the interferometer cell is shown in Fig. 2, and a set of adsorption cells is shown in Fig. 3. Finally the complete setup is demonstrated in the photograph Fig. 4. A detailed description is found in the papers referred

to above. The self-recording apparatus of Claesson (1944) will be described somewhat more in detail, as the original publication may not be available to some readers of the present paper. From Claesson's description we quote the following:

"The construction of a self-registering apparatus for the purpose mentioned really involves two problems, namely the registering of the refractive index and that of the volume or weight of the solution with accuracies of  $10^{-5}$  and 0.1 ml., respectively. These two registering arrangements should then be co-ordinated so as to give only one curve on a photographic paper

The registering of the refractive index was arranged in the following manner (see Fig. 5). The solution, after having passed the adsorbent,

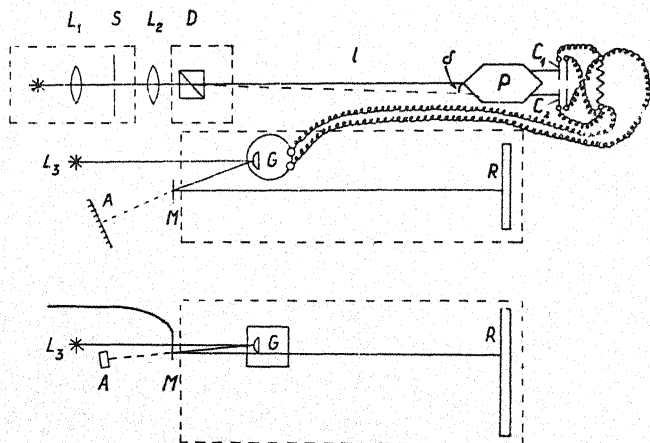


FIG. 5. Diagram of Claesson's self-recording refractive index adsorption analysis apparatus.

enters one of the halves of a double prism ( $D$ ). The other half is filled with solvent. The angle between the halves is  $45^\circ$ . A beam of light from the slit ( $S$ ) travels through the prism and falls on the edge of a hexagonal prism ( $P$ ) of glass, where it is split into two parts, each falling on a blocking-layer photo-element, ( $C_1$ ) and ( $C_2$ ). The two elements are connected with a galvanometer ( $G$ ) in such a way that no current passes through it when the elements are equally illuminated.

We now suppose that the refractive index of the solution in one of the halves ( $D$ ) changes by  $\Delta\mu$ , causing the light beam to move a distance  $\delta$ . The following relation holds

$$\Delta\mu = \frac{\delta}{l} \quad (2)$$

where  $l$  is the distance between the prisms. The movement of the light beam gives rise to unequal illumination of the elements ( $C_1$ ) and ( $C_2$ ) and the galvanometer makes a deflection which can be registered on the photographic paper ( $R$ ).

In the expressions for the amount of light  $F$  which moves from one of the elements to the other when the light beam moves through  $\delta$  following the change  $\Delta\mu$  in the refractive index, we use the following notation: the galvanometer deflection  $n$ , the illuminating power of the lamp  $L$ , the current from the photo-cells  $I$  (which is assumed to be proportional to the amount of light  $F$ ;  $k$ ,  $k_1$ ,  $k_2$  and  $K = k \cdot k_1 \cdot k_2$  are constants. We then have

$$\begin{aligned} F &= k \cdot L \cdot \delta \\ I &= k_1 \cdot F \\ n &= k_2 \cdot I \\ \text{Thus } n &= k \cdot k_1 \cdot k_2 \cdot L \cdot \delta = K \cdot L \cdot \delta \end{aligned} \quad (3)$$

and from (2) we have

$$n = K \cdot L \cdot l \cdot \Delta\mu$$

or

$$\Delta\mu = \frac{1}{l} \cdot \frac{1}{K \cdot L} \cdot n. \quad (4)$$

From (4) it is found that  $\Delta\mu$  is directly proportional to the galvano-

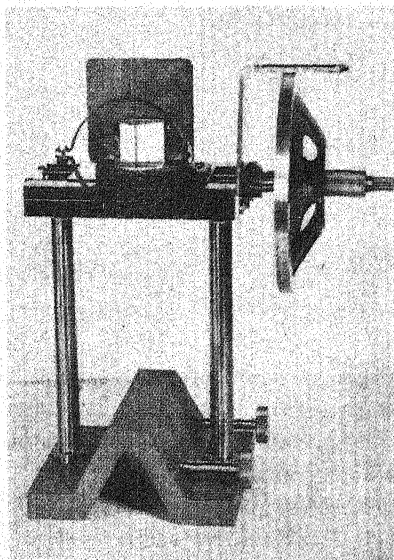


FIG. 6. Movable prism for the self-recording apparatus.

meter deflection  $n$  if the photo-elements have linear characteristics (i.e., if  $k_1$  is an absolute constant). It is also seen from (2) that if  $l$  is chosen as 1000 mm. and  $\Delta\mu$  as  $10^{-5}$  the relation gives  $\delta = 0.01$  mm., and in order to make the galvanometer deflection 1 mm. the amplifying factor  $KL$  must have the value of 100. These two requirements may be satisfied by blocking-layer photo-elements if the prescriptions concerning their coupling given by Wood are observed. As photo-element a "Differenzial Photoelement S 60" from B. Lange has been employed. The galvanometer was a "Moll Original" type D (resistance 65  $\Omega$  and sensibility  $2.4 : 10^{-9}$  amp./mm.). The galvanometer was shunted by a 35  $\Omega$  resistance.

The prism ( $P$ ) and the elements ( $C_1$ ) and ( $C_2$ ) are mounted on a slide that may be moved at right angles to the light beam by means of a micrometer screw, which can be read to 0.001 mm. (see Fig. 6). Equation (3) was used in showing that the galvanometer deflections are directly proportional to the difference in the light-intensities between the elements ( $C_1$ ) and ( $C_2$ ).

To give  $\delta$  differential values, the slide was moved by the micrometer screw. The corresponding galvanometer deflections were observed and the results showed completely linear relationship.

The author decided to weigh the solution that had passed through the double prism instead of measuring its volume. The choice of the balance was of great importance, as very different amounts of solutions are used in different experiments (from 10 g. to 1000 g.), and so the balance must be easy to replace by others with different sensitivities. Simple straight springs seem to be best suited. The spring is fastened horizontally at one end, while at the other hangs a vessel into which the solution drops. The balance is damped by a small metal plate dipping into paraffin oil. For the loaded spring the well-known formula

$$\tan \alpha = \text{constant} \cdot P$$

holds, where  $\alpha$  is the angle between the free end and the horizontal plane and  $P$  is the load. At the free end of the spring a mirror is fastened, the deflection being registered as usual by means of a light spot on a scale. As the arc  $\tan$  correction does not enter, the deflection is directly proportional to the load.

The recording devices for refractive index ( $\Delta\mu$ ) and weight ( $g$ ) are co-ordinated in the usual way with crossed mirrors and thus  $\Delta\mu$  is registered as a function of  $g$ . The mirror on the balance moves at right angles to the galvanometer mirror (see Fig. 5). A narrow beam of light from the illuminating device ( $L_3$ ) first reaches the galvanometer mirror and then the balance mirror ( $M$ ) and forms a point image on the photo-

graphic paper (*R*). When the refractive index changes, the image moves horizontally, and when the weight increases it moves vertically, and so it describes a curve of the type desired with  $\Delta\mu$  as a function of *g*. The photographic paper has a size  $21 \times 29$  cm. The whole apparatus and registering devices are surrounded by lightproof housings. The apparatus may even be used in broad daylight. To render it possible to observe how the experiments proceed, the mirror (*M*) is half-reflecting and an image falls on the scale (*A*). The double prism (*D*) is immersed in a thermostat.<sup>1</sup>

The filter for the adsorbent and the other arrangements are the same as in the earlier apparatus but here much larger filters can be used with volumes of 400 cm.<sup>3</sup>.

The volume in the double prism available for the solution is 0.23 ml., which is small enough to avoid considerable volume-lag.

Experiments with several different classes of substances (among others saccharides, amino acids and peptides, fatty acids) have generally been performed according to one of the following three principal methods, namely frontal analysis, elution analysis and displacement analysis.

#### IV. FRONTAL ANALYSIS

In this method the solution is allowed to pass the adsorption column, and the concentration distribution at the adsorption front is analyzed. The different components in a mixture appear successively with sharp boundaries in the solution leaving the adsorption cell. An example is given in the curve Fig. 7, from an experiment with a solution containing

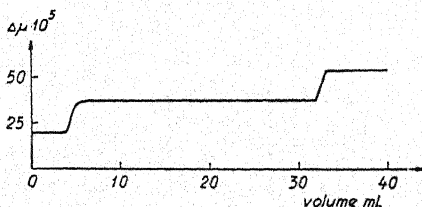


Fig. 7. Frontal analysis of a mixture of alanine and leucine.

alanine (0.159 mg. N per ml.) and leucine (0.104 mg. N per ml.) on 1.5 g. Norite P 3 Spezial (active carbon). The horizontal axis shows the volume of solution running through the cell, the vertical axis the refractive index increment as read in the interferometer. Up to about 4 ml. only water appears. Then suddenly the concentration rises and a layer of pure alanine appears, followed at about 32 ml. by a new boundary

<sup>1</sup> The complete apparatus is now manufactured by LKB Produkter Fabriks AB, Alvik, Stockholm.

when the leucine begins to appear. From now on the composition of the solution is identical with the original, and the adsorption column is saturated. The volume of solution flowing through before a substance appears is called the retention volume (earlier retardation volume). Determination of nitrogen in the alanine layer gave a concentration of 0.161 mg. N per ml. (*cf.* above) and thus a determination of the concentration in each step in a front analysis diagram — either optically or analytically — allows the determination of the composition of the original solution. However, as has been stated in earlier communications, the necessary condition is that there is no mutual displacement of the substances on the adsorbent. (The reader is referred to the treatment in the author's paper in *Advances in Colloid Science* I, 81, 1942, and to the detailed discussion in a paper by Claesson, 1945). This is the case if the components are weakly adsorbed, as in the instance above. Generally, however, the displacement will give rise to an abnormally high concentration of the first step. Corrections may be applied in special cases (Claesson, 1945, 1946) but require the knowledge of certain constants which makes them practicable mainly for substances in homologous series. Another drawback of this procedure is the comparatively large quantities of material required. However, Claesson has successfully used this method for analyzing mixtures of fatty acids and of hydrocarbons.

## V. ELUTION ANALYSIS

Elution analysis corresponds more closely to the normal procedure in chromatographic analysis. A sample of the solution to be analyzed is pressed into the column and is washed through by addition of large quantities of solvent. Fig. 8 shows a diagram of an elution analysis

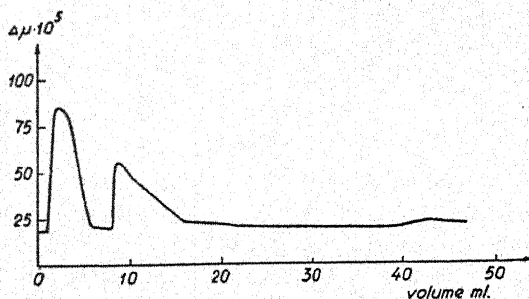


FIG. 8. Elution analysis of a mixture of alanine, valine and leucine.

as obtained by the micro-interferometric method with a sample containing alanine (1.55 mg. N), valine (1.06 mg. N), and leucine (0.97 mg. N)



using active carbon Carboraffin C as an adsorbent and only water for the elution. The analysis of the portions corresponding to the three peaks in the figure gave 1.59, 1.05 and 0.82 mg. N, respectively, confirming that leucine is not quantitatively eluted, at least not with the limited amount of water used in this experiment. The diagram shows the merits and the disadvantages of this procedure. One obtains a complete separation, but for the more strongly adsorbed substances the tailing is so marked that the optical method becomes useless for the observation of the zones. In the case mentioned, the leucine can be eluted completely by washing out with for example 5% acetic acid but an optical control of this procedure is not possible and hardly necessary.

## VI. DISPLACEMENT ANALYSIS

It was pointed out by the author (Tiselius, 1943) that if strongly adsorbed substances are used for the displacement in adsorption analysis, the development takes place in a way which is in principle different from the ordinary elution. The displacing substance (*D*) behaves as in ordinary frontal analysis (see above) but will push the other adsorbed components *A, B, C* just before its adsorption front through the column. *A, B* and *C* then gradually form sharply separated zones, each containing one component and in close contact with each other and the front of *D*. An example as recorded by the interferometric method is shown in Fig. 9 with a diagram from the analysis of a mixture of leucyl-glycine

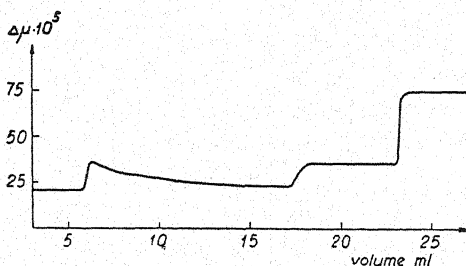


FIG. 9. Displacement analysis of a mixture of leucyl-glycine and leucyl-glycyl-leucine, with 0.25% phenol as displacing solution.

(0.75 mg. N per ml.) and leucyl-glycyl-glycine (0.85 mg. N per ml.) on 0.25 g. Carboraffin C with a 0.25% phenol as displacing solution. The phenol front is the large step to the right in the figure. The middle step is the leucyl-glycyl-glycine and the lowest step the leucyl-glycine. The latter has not yet reached the stationary state—if a larger column is used also this step becomes quite even. Contrary to the elution method, the eluent is not found in any peptide zones. A simple consideration shows that the *height* of each step, including that of the displacing

substance, is independent of the amount of substance but is determined only by the adsorption affinity, whereas the *length* is proportional to the amount. In the stationary state all zones must migrate through the column with equal velocity (equal to that of the front of the displacing substance). It was stated above that the rate of migration of a zone is a function of the adsorption coefficient. In Fig. 10, a number of adsorption

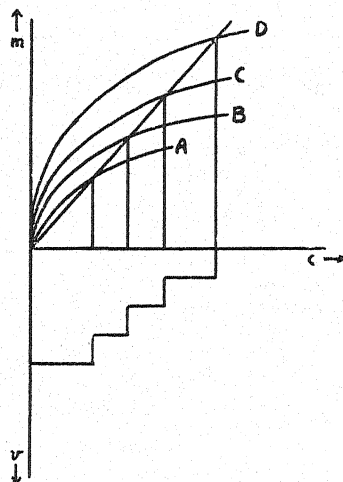


FIG. 10. Diagram illustrating the principle of displacement adsorption analysis.

isotherms have been drawn for substances A, B and C, and the displacing substance D. A straight line intersects the isotherms at points corresponding to identical adsorption coefficients. These points therefore represent the stationary concentrations during the development and the shape of adsorption analysis curve can be deduced as shown in the lower half of the figure in which the volume axis points downwards. The stationary concentrations for the most weakly adsorbed substances are very low and are not established unless a very large column is used.

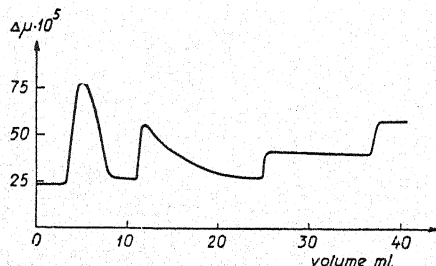


FIG. 11. Displacement analysis of a mixture of valine (peak to the left), leucine (next peak), and methionine (first step). Displacing agent: 0.5% aqueous solution of ethyl acetate (second step).

Under ordinary conditions such components appear as peaks in the diagram in front of the displacement "step" curve as shown in Fig. 11 identical with those obtained by elution analysis as described above.

The advantage of this type of adsorption analysis evidently lies in the stability of the zones: theoretically at least one should be able to use as large columns as desired and therefore to carry out also very difficult separations. The main drawback of the elution method — the tailing — is eliminated. The fact that the different zones are in close contact is a difficulty which, however, may be eliminated by interposing substances of intermediate adsorption and of a different chemical nature so that they may easily be removed afterwards. Examples of the application of this procedure may be found in a paper by Hahn and the author (Tiselius and Hahn, 1943), in which various saccharides were separated, and some experiments on amino acids and peptides already mentioned. In case of strong adsorption, the affinity of the adsorbent has to be reduced by pre-treatment with suitable substances as described in the last mentioned paper. Nevertheless, losses are sometimes serious in this method. The experience so far seems to show that with the adsorbents now available the displacement method is applicable chiefly for mixtures of closely related substances, which are able to displace each other completely. However, for fatty acids in organic solvents on carbon the method has failed, but in the separation of gaseous hydrocarbons it seems quite satisfactory (Claesson, 1946).

It should be noted that the displacement of inorganic ions in an exchange adsorption column by addition of dilute acid should proceed according to the principles described above. This has been confirmed by some experiments of Brattsten and the author (not yet published). In most cases of ordinary chromatography it may be difficult to say if elution or displacement development is used. If the displacing solution is applied in high concentration or if it is added too fast, there is insufficient time for a stationary state to be established, and the front of the displacer will overrun the other zones and a sort of mixed elution and displacement will occur. This is also the case if coarse grained adsorbent material is used, in which diffusion into the interior requires too much time to allow the adsorption equilibrium to be established. Nevertheless, it is useful to know the above three ideal types of adsorption analysis even if they cannot always be realized experimentally.

It is evident from the foregoing that the optical methods for adsorption analysis as described above (pp. 70-78) are not so well suited for the elution analysis as for frontal analysis and for the displacement method. The change of solvent often necessary in elution development is also inconvenient with the interferometric method as the measuring interval

is narrow and may have to be shifted by special arrangements if the refractive index of a new solvent is markedly different. In elution analysis one can often use with advantage sensitive specific reagents to control the separation in the filtrate (*e.g.*, the ninhydrin reaction for amino acids) if the various components cannot be observed in the column. If this is possible—either by direct or indirect methods—it is of great advantage. The concentration of adsorbed material in a zone is naturally higher on the column than in an eluate and it should therefore in general be much easier to make direct observations of elutions on the column, if suitable observation methods are available (see below). There are, of course, a number of other possibilities for the continuous analysis of the filtrate: light absorption, conductivity, and potentiometric measurements, and others. As far as the author knows, these methods have not been tried out. The conductivity method might be of value in ionic exchange adsorption.

#### VII. LOCATION OF AMINO ACIDS AND PEPTIDES ON THE COLUMN

Of the methods for locating uncolored substances on an adsorption column (see Strain, 1942, p. 71) only a few have been used for amino acids and peptides. Waldschmidt-Leitz and Turba (1940) observed that the zones formed when a peptide mixture was adsorbed on Filtrol-Neutrol (a bleaching clay) were a little lighter in color than the original adsorbent and could thus follow the separation of some peptides. The method is not very sensitive, and in his later work Turba seems to prefer other methods. Karrer, Keller, and Szony (1943) transferred methyl esters of glycine, alanine, leucine and valine with *N-p*-phenyl-azo-benzoyl chloride in pyridine to colored acyl compounds which were separated on a column of zinc carbonate in benzene-ligroin solution. The quantitative application is, however, difficult, as the acylation process is not uniform.

Schramm and Primosigh (1944) have recently recommended a number of methods for making the adsorption of amino acids on aluminum oxide columns visible. The simplest method is to make the amino acids form colored compounds in the column, by allowing a dilute copper acetate solution or a solution of chinone to flow through. Better results were obtained by a sort of displacement method, in which the column is first colored with a weak solution of a dye of suitable adsorption affinity, for example bromothymolblue, methyl red, nitro- or dinitrophenols. The amino acid zone appears colorless against the colored background.

#### VIII. THE PARTITION PRINCIPLE IN ADSORPTION ANALYSIS

The chief difficulty of the elution analysis depends upon the tailing phenomenon which in its turn is a consequence of the non-linearity of

the adsorption isotherm, as discussed above. The partition of a solute between two immiscible solvents follows Henry's law, that is, the concentrations in the two phases are proportional in a fixed ratio. This may occur also in ordinary adsorption, and according to Langmuir should be the rule at low concentration. Adsorption analysis performed under such conditions should make it possible to use elution development without too much spreading of the zones. Martin and Synge (1941,2) were the first to make use of this principle in the separation of acetylated amino acids, using water-moistened silica gel as one phase and organic solvents as the other. This remarkable investigation was a further development of their earlier attempts to use successive extraction methods for such separations (1941,1). Martin and Synge showed that the partition chromatography could be regarded as an extreme case of an extraction fractionation for which the number of theoretical plates in the column is very large. If  $\alpha$  in the equation for the rate of migration of a zone in an adsorption column (p. 69) is the partition coefficient instead of the adsorption coefficient, the same equation is valid for partition chromatograms. There is hardly any difference in principle between ordinary and partition chromatography. An approximately linear adsorption isotherm is found in many cases of weak adsorption. On the other hand, the partition coefficient may vary, decreasing with increasing adsorption coefficient. The main reason for this is, as in adsorption, the limited space available for the solute in one phase.

In the partition chromatographic method of Martin and Synge, the zones of amino acids could be made visible with the aid of a suitable acid-base indicator in the water phase. The method and its applications will not be treated further here, as these authors recently have given a review on the subject (1945).

Evidently the partition principle is of advantage also for frontal analysis and it would make a correction for displacement effects unnecessary. In partition there should be no displacement effects under ideal conditions. For this reason displacement development cannot be used in that case.

## IX. THEORIES OF ADSORPTION ANALYSIS

In addition to the papers referred to above several others have recently dealt with the theoretical treatment of the processes during the migration of the zones in an adsorption column in order to estimate the possibilities of the separation of different substances. The concentration distribution for different quantities of the eluent when two components migrate through an adsorption column can be calculated if the adsorption isotherms of both components are known as well as the mutual influence

of each component on the other's adsorption. The Langmuir theory would give an expression for both, but it is doubtful if this theory is valid in solutions. Thus, so far there has not been much practical application of the theoretical results obtained, and the reader is only referred to some recent additional papers by the following authors: Wilson (1940), de Vault (1943), Weiss (1943), Weil-Malherbe (1943), Offord and Weiss (1945), Glückauf (1945). The observation method described above and the type of diagrams obtained as shown in Figs. 7-9 and 11 should be particularly well suited for further experimental tests.

### X. MOLECULAR ADSORPTION ANALYSIS

Measurements by Abderhalden and Fodor (1919), Negelein (1923), Ito (1930, 1932, 1936), Wunderly (1934) demonstrated that amino acids were adsorbed more or less strongly by active carbon preparations. The author (1941) extended these measurements using the determinations of the retention volume by the methods described above as a convenient means of comparing the amount of adsorption for the different substances. Table I shows such a series of measurements on a particular carbon. Some peptides are also included. Other active carbons investigated showed somewhat smaller or greater effects but the order in Table I was not changed.

TABLE I

RETENTION VOLUMES IN THE ADSORPTION OF SOME AMINO ACIDS AND PEPTIDES  
ON ACTIVE CARBON (CARBO ACTIV SCHERING)  
0.5% aqueous solutions.

Substance	Ret. vol. ml./g. carbon	Substance	Ret. vol. ml./g. carbon
Alanine	0.3	Glycyl-glycine	3.5
Hydroxyproline	2.0	Leucyl-glycine	18.2
Proline	2.5	Leucyl-glycyl-glycine	29.8
Valine	3.2	Glycyl-alanine	4.0
Leucine	7.7	Valyl-alanine	22.0
Isoleucine	9.2	Alanyl-leucyl-glycine	34.4
Methionine	12.4	Glycyl-leucyl-alanine	42.5
Histidine	15.0	Glycyl-leucyl-glycine	38.0
Arginine	40.4		
Tryptophan	76.5		
Phenylalanine	62.5		

Measurements of the adsorption of amino acids on charcoal have also been made recently by Cheldelin and Williams (1942). Thus very marked differences are observed and theoretically at least the chances of separating amino acids and peptides on such adsorbents should be good.

It is seen that tryptophan and phenylalanine are most strongly adsorbed: to these one should add tyrosine, which however is not included in the table as it is not sufficiently soluble at neutral reaction to give a concentration of 0.5%. As a matter of fact it is possible, as Schramm and Primosigh (1943) have shown, to wash out all other amino acids from a column with 5% acetic acid, leaving the three last mentioned acids in the column from which they may be eluted quantitatively with 5% phenol in 20% acetic acid. This procedure has also been tried in the author's laboratory with good results. It is suitable as the first step in the separation of amino acids from a protein hydrolyzate. If the hydrolysis is incomplete the elution of the aliphatic compounds may not be quantitative as some peptides are bound as strongly as the aromatic amino acids.

No doubt even the smaller differences in retention volume for the other acids in Table I may in favorable cases be utilized for a separation according to this simple "washing out" procedure. Thus Turba, Richter and Kuchar (1943) reported a separation of methionine/valine on 6 g. carbon, where 95% of the methionine is eluted with 400 ml. water, and of valine/leucine (5 mg. each) on 4 g. carbon, elution with 300 ml. *M*/15 phosphate buffer, pH 5.6 (98% of the valine eluted). Wachtel and Cassidy (1943) separated a quaternary mixture (tyrosine, phenylalanine, leucine and glycine) on Darco G-60 charcoal. These authors demonstrated that the amino acids may undergo some deamination or oxidation during the process as was to be expected from earlier observations on the catalytic influence of charcoal by Warburg and Negelein and by Baur and Wunderly (for literature references see the paper by Wachtel and Cassidy, 1943, p. 667). The author (1941) as well as Schramm and Primosigh (1943) also paid attention to such effects. If, as suggested by the author (1941), the charcoal is poisoned by treatment with potassium cyanide and if one works rapidly there is hardly any risk of such effects playing any important role.

From the experience so far it appears likely to the author that the main application of charcoal in amino acid analysis, according to the "all or none" method, will be for the removal of tyrosine, tryptophan and phenylalanine from hydrolyzates, prior to the separation on other adsorbents. It is true that other separations are possible but it seems that those can be made more safely with for example the exchange adsorbents mentioned below. With interferometric or similar methods for following the separation process in detail charcoal finds a much wider application for more difficult separations of mixtures of neutral amino acids and also of peptides. Examples have already been shown in the Figs. 7-11 above.

Schramm and Primosigh use Schering's granulated active charcoal

which gives very little resistance of flow in the column. The author prefers using a finely ground preparation (as commonly used for the decolorization of solutions) and uses a pressure of about 0.5-1 atm. to obtain a suitable rate of flow through a column about 10-20 mm. high. In such a column, equilibrium is reached much more rapidly than in one made up of coarse grained charcoal. This is a necessary condition for the successful application of any observation method for the control of the separation and also for obtaining sharp separations.

The procedure recommended by Schramm and Primosigh includes a pre-treatment of the column with 5% acetic acid. Without this procedure, part of the amino acids remain firmly bound to the adsorbent. This has also been a quite general experience in the numerous experiments by the author and his collaborators, using active charcoal as adsorbent. In the separation of the higher saccharides a partial saturation of the charcoal with phenol or ephedrine was found necessary (Tiselius and Hahn, 1943).

Separations of methionine, leucine, valine, and alanine using 0.5% ethyl acetate as displacing solution in the interferometric apparatus have been described by the author (Tiselius, 1944) together with several other examples of separations of the neutral amino acids on charcoal. Also peptides may be separated (see Fig. 9 above, taken from the same work). The possibilities of the method for this class of substances have not yet been fully explored. It is quite evident, however, from the experience in the author's laboratory, that the differences between different peptides are large and that a separation in many cases is possible. The losses, however, tend to become serious with peptides with more than two or three amino acid residues, probably on account of the above mentioned irreversible binding. A further study of suitable methods for pre-treatment to give a partially saturated adsorbent is necessary. The author has tried to treat the charcoal in a vacuum with varying quantities of naphthalene and obtained a number of "graded" preparations of weaker adsorption affinity, which however did not give satisfactory separation diagrams, probably on account of a markedly inhomogeneous adsorption. There is no doubt, however, that much could be gained by suitable methods for reducing the *adsorption affinity* as evenly as possible (or better: most markedly for the spots of highest affinity) without reducing too much the available *adsorption area*.

For strongly adsorbed substances it is not so easy to find suitable displacing agents. These should naturally have higher retention volumes than the substances to be displaced. The following substances have been tried (the retention volume refers to 0.5% solutions on the same charcoal as used in Table I above).



TABLE II  
RETENTION VOLUMES FOR DISPLACING AGENTS

Substance	Ret. vol. ml./g. carbon
Saccharose	23
Methyl-propyl-ketone	31
Ethyl acetate	32
Pyridine	33
Raffinose	46
Phenol	53
Ephedrine	83
Picric acid	83

If there was a general competition about all available adsorption sites any strongly adsorbed substance should displace any less strongly adsorbed. Such is however not the case. Phenol, for example, is sometimes less effective than ethyl acetate. Evidently specific effects, of which very little is known so far, may interfere.

#### XI. IONIC EXCHANGE ADSORPTION ANALYSIS

The base exchange type of adsorption, well known in permutites and similar materials used in water-softening plants, plays an increasing role for the separation of amino acids, peptides and other organic substances. Both cationic and anionic exchangers are known, and the field is developing rapidly since the introduction of the synthetic exchange resins, in which the characteristic properties may be varied almost at will. (For a recent review of these "organolites" see Myers *et. al.*, 1941, 1942) Naturally in the first place, substances of a more or less pronounced electrochemical character lend themselves to work of this kind, that is in our case diamino-monocarboxylic acids and monoamino-dicarboxylic acids and peptides with such residues. By addition of other substances, however, (see below) it is possible to modify the electrochemical properties also of the neutral amino acids so that some of these may show distinct adsorption properties towards exchange adsorbents. It must also be remembered that it is difficult to isolate one type of effect from another and that very likely some molecular adsorption plays a role also with the typical exchange adsorbents. The relative ease with which the adsorption may be influenced by a change in the ionic medium, especially as regards the hydrogen ions, is, however, characteristic and of great practical value in the separation and elution procedures.

Most of the natural ion exchangers are cation exchangers, for example aluminum oxide, aluminum hydroxide, silica gel and zeolites. Treatment of aluminum oxide with acid, however, transforms it to an anion ex-

changer. One must therefore remember that exchange adsorbents do not have a fixed electrochemical character. This is particularly important in practical work as one often has to make use of different pH values, and this will change the dissociation not only of the substance to be adsorbed but also the adsorbent itself. This effect should be less marked for the strongly acid synthetic resins with  $\text{SO}_3\text{H}$  groups but should be larger for the resins with carboxyl groups and is most pronounced with amphoteric adsorbents like aluminum oxide, which by treatment with acid is transformed from a cation to an anion exchanger.

Another characteristic property of this type of adsorption is the marked dependence of electrolyte concentration, which is particularly noticeable for adsorbents of the aluminum oxide type. This complicates the situation because it may make it difficult to apply buffer solutions which otherwise would be desirable to maintain a defined pH throughout the separation. There are not yet any detailed systematic investigations on the adsorption of ampholytes on exchange adsorbents under sufficiently varied conditions (particularly pH and electrolytes should be varied), but a large amount of empirical data have been collected in connection with the recent attempts to use these adsorbents for the separation of amino acids, showing the differences in behavior for different amino acids. The reader is referred especially to the recent papers by Englis and Fiess (1944) and by Cleaver, Hardy and Cassidy (1945).

The application of zeolites for preparative separations of amino acids was already described by Whitehorn in 1923. In 1940, Waldschmidt-Leitz and Turba published a very interesting paper on the separation of peptide mixtures (obtained by enzymatic splitting of clupein) by chromatography on a sort of bleaching clay called Filtrol-Neutrol, which evidently is a base exchanging substance. The position of the zones could be observed as described on p. 81 on account of a slight change in color of the adsorbent at the site of the zones. The adsorption affinity was dependent upon the amount of arginine in the peptides. In a later work (1941) the same authors and Ratzer separated a number of such clupein peptide fractions in sufficient amount to analyze them and show that each of the zones had different  $\text{N}:\text{NH}_2$  ratios. Naturally each zone may contain a mixture of peptides which may be different in other respects than those which determine the adsorption. Turba has continued this work in a number of papers with the separation of amino acids as the main object. He first continued to use the cation-exchanging bleaching clays and was able to make quantitative separations of mono- and diamino acids and also, for example, of arginine and histidine (1941). The clays are very effective as adsorbents but tend to offer too large resistance in the percolation. The use of aluminum oxide, introduced by

Wieland (1942), as a cation exchanger for the separation of the basic amino acids was an improvement in this respect and also because it was easier to obtain the adsorbent pure. This use of aluminum oxide originated in the well known work of Schwab and his collaborators on the separation of inorganic ions on aluminum oxide. It was known from this work that treatment with strong acids converts the aluminum oxide to an anion exchanger. This is capable of adsorbing and separating dicarboxylic amino acids (Wieland, 1942, 1943, Wieland and Wirth, 1943). Also Turba and Richter (1942) used the acid aluminum oxide for the separation of glutamic and aspartic acids in acetate buffers. Schramm and Primosigh (1944,1) prefer silica gel to other possible adsorbents (including the synthetic resins) for the binding of the diamino acids, on account of easy elution and good permeability. The author's own attempts to use this adsorbent have not been so successful, and it is evident that the mode of preparation plays a decisive role.

The invention of Adams and Holmes (1935) of the synthetic organic exchange resins already mentioned above opened up new possibilities also in this field which was soon realized by a number of authors. In U.S.A. and England mainly the resins Amerlites IR-100, and IR-4 (Resinous Products and Chemical Company, Philadelphia) have been used, of which the first is a cation, the latter an anion exchanger (Block, 1942; Platt and Glock, 1942; Cannan, 1944; Englis and Fiess, 1944; Cleaver, Hardy and Cassidy, 1945). Simultaneously and apparently independently, German workers applied the analogous resins Wofatit KS, K, C, and P (made by I. G. Farbenindustrie A.G., Wolfen), of which KS and K are cation exchangers with  $\text{SO}_3\text{H}$  groups, C also a cation exchanger but with  $\text{COOH}$  groups, whereas P is an anion exchanger, (Freudenberg, Walch and Molter, 1942; Turba, Richter and Kuchar, 1943; Wieland, 1944).

The resins have a much greater capacity than aluminum oxide. According to Wieland (1942, 1944) 1 g. Wofatit C binds at least 100 mg. histidine, whereas 1 g. aluminum oxide binds only about 2 mg. lysine hydrochloride. Still more important, however, is their greater stability. Columns of clays, fuller's earths, and aluminum oxide gradually change their capacity and grow less and less permeable, whereas the resin filters may keep almost indefinitely. They may, however, gradually give off some organic material, and it is necessary to pretreat them thoroughly by repeated washing with acids and alkali (see for example Cleaver *et al.*, 1945). Details about their adsorption properties towards different amino acids are given in the papers cited above. Only some general rules will be mentioned here. The cationic exchangers are mostly used in neutral or almost neutral solution after treatment with HCl and washing out

with water. The application of buffer solutions offers definite advantages, but the cations tend to reduce the adsorption to a certain extent. The strongly acid resins, containing  $\text{SO}_3\text{H}$  groups will bind all amino acids as cations, whereas the carboxyl resins (e.g., Wofatit *C*) only binds the diamino acids, and the neutral and the dicarboxylic acids will run through. In presence of potassium ions, the affinity of Wofatit *C* to histidine is so much reduced so that this amino acid may be separated from arginine and lysine this way (Wieland, 1944; see also Cleaver *et al.* 1945). The elution is brought about by addition of acid (for example 1*N* HCl). The diamino acids are difficult to elute from the strongly acid  $\text{SO}_3\text{H}$  resins. A moderation and finer gradation of the adsorption affinities in various cases might be possible by suitable combination of defined pH and concentration of different inorganic cations.

By analogy, the anion exchangers like Amberlite *IR-4* should bind preferentially the dicarboxylic acids. This is also the case. A mixture of amino acids and hydrochloric acid, which is stirred for 1 to 3 hours with sufficient resin *IR-4* to give a final pH of 6 to 7, will adsorb completely the acidic amino acids and leave the neutral and basic amino acids unaffected (Cannan 1944, Cleaver *et al.*, 1945). On the pure fraction eluted with HCl from the resin, Cannan determined the aspartic and glutamic acids separately by a combination of electrometric titrations in water and formaldehyde solutions and a gasometric ninhydrin estimation—a method well suited for work on a semi-micro scale.

If the Sakaguchi reaction or one of its modifications is used for the determination of arginine we thus may, by the separations described, analyze for arginine, lysine, histidine, aspartic and glutamic acids with quantities of protein much less than 1 g. and using exclusively adsorption methods for the separation. Some preliminary experiments in the author's laboratory show that it might be possible to separate arginine and lysine in the interferometric apparatus described above and the same is probably true for mixtures of aspartic and glutamic acids.

The neutral amino acids are adsorbed on the strongly acid resins, as has already been mentioned, but there does not seem to be any marked differentiation. An exception is tryptophan which according to Turba *et al.* (1943) is adsorbed selectively on Wofatit *M*, pretreated with *M*/5 acetic acid. The methods known from amino acid titration to change the electrochemical character of neutral amino acids have been applied also to modify their adsorption properties. Thus Schramm and Primosigh (1943, 1944) by treatment of a mixture of neutral amino acids with 10% formaldehyde and thereby masking the amino groups, could increase the affinity of serine, glycine, threonine and cysteine to an anionic exchanger (acid-treated aluminum oxide) so that these acids could be

separated from the rest in the mixture. Addition of alcohol is another way of modifying the electrolytic dissociation of neutral amino acids. Turba *et al.* (1943) describe several successful separations of neutral amino acids on Filtrol-Neutrol and aluminum oxide in 50% alcohol. Thus proline, glycine and alanine (5 mg. of each) were adsorbed, whereas valine and leucine passed through a filter of 30 g. Filtrol-Neutrol. Cystine is bound in 50% alcoholic solution on aluminum oxide pretreated with acetic acid and may thus be separated from all the other neutral amino acids including methionine. The application of these and similar methods for modifying the adsorption behavior also on organolites is now being investigated in the author's laboratory.

The "elution" of amino acids and other cations from a cation exchanger with acid should be a typical displacement reaction. Measurements with the optical methods show that this is the case, if the process is carried out slowly on a finely powdered material. Otherwise the acid will overrun the other ions, and an ordinary elution results. The resin exchangers seem to work more slowly than the aluminum oxide. It is not yet clear if the development of the chromatograms on the resinous exchangers proceeds sufficiently regularly to make an optical observation worthwhile. If such would be the case, however, there should be great possibilities in further analyzing mixtures of neutral amino acids also on these adsorbents.

Thus a great many alternative methods of conducting the adsorption analysis offer themselves in this field. To these one should add the valuable procedures for "partition chromatography" which have been described in an earlier review by Martin and Synge in the preceding volume of *Advances of Protein Chemistry*. As was emphasized in the introduction, it is not yet possible to make definite recommendations for the best working procedures, as the different possibilities have not been tried out under sufficiently varied conditions. One must also remember that, even if the accurate and convenient analysis of protein hydrolyzates on a semi-micro scale is the immediate goal, the methods should also be judged from the standpoint of their potentialities for an extension into the field of peptide separation and analysis—a field which should be of the utmost importance for our knowledge of the fine structure of proteins.

It is thus too early to give a suitable combination of the above methods to a definite "scheme" for the analysis of protein hydrolyzates. The reader is referred to the papers of Wieland (1942), Turba, Richter and Kuchar (1943), Schramm and Primosigh (1944) for various suggestions. The latter authors emphasize the importance of using as small quantities of adsorbent as possible, to avoid unnecessarily large volumes of wash-

ing solutions, and the necessity of avoiding accumulation of salts, which may seriously hamper the use of certain adsorbents (silica gel, aluminum oxide). The irregular behavior of cystine also is a complication. This amino acid is easily oxidized to the corresponding sulphonic acid, which shows quite different adsorption properties. To prevent this, Schramm *et al.* recommend to make the adsorption in presence of  $H_2S$ .

Drake and Hagdahl in the author's laboratory (to be published shortly) start, following Schramm *et al.* (1944), by adsorbing the aromatic amino acids on active charcoal, pretreated with 5% acetic acid. All other amino acids may then be washed out with 5% acetic acid. This solution is directly passed through a  $COOH$  resin (Wofatit C) which binds the diamino acids. It is better not to use a  $SO_3H$  resin here, as difficulties then may arise in the elution of the diamino acids. The percolate from Wofatit C is now immediately led through a  $SO_3H$  resin (Wofatit K) filter which binds all the remaining amino acids but will let all the salts through. From the charcoal filter the aromatic amino acids are eluted quantitatively by the method of Schramm *et al.* with 5% phenol in 20% acetic acid. The diamino acids are recovered from the C filter by treatment with 1N HCl, and all the other amino acids are obtained free from salts by treating the K filter with a 5% aqueous pyridine solution, and then the three percolates are evaporated. The last mentioned fraction may be dissolved in 0.01 N acetate buffer of pH 3.3 and the dicarboxylic acids adsorbed on acid aluminum oxide according to Wieland and Wirth (1943). One may now also use an anion exchanging resin, as employed by Cannan (1944). The further fractionation of the neutral amino acids may follow any of the above mentioned alternative ways. If resins can be used also in the further steps, or if charcoal and optical methods of observation can be used it may be unnecessary to apply the treatment with the K resin to remove the salts. The above scheme makes no use of the partition methods. It is quite obvious, however, that these should offer great possibilities of further improving the procedure.

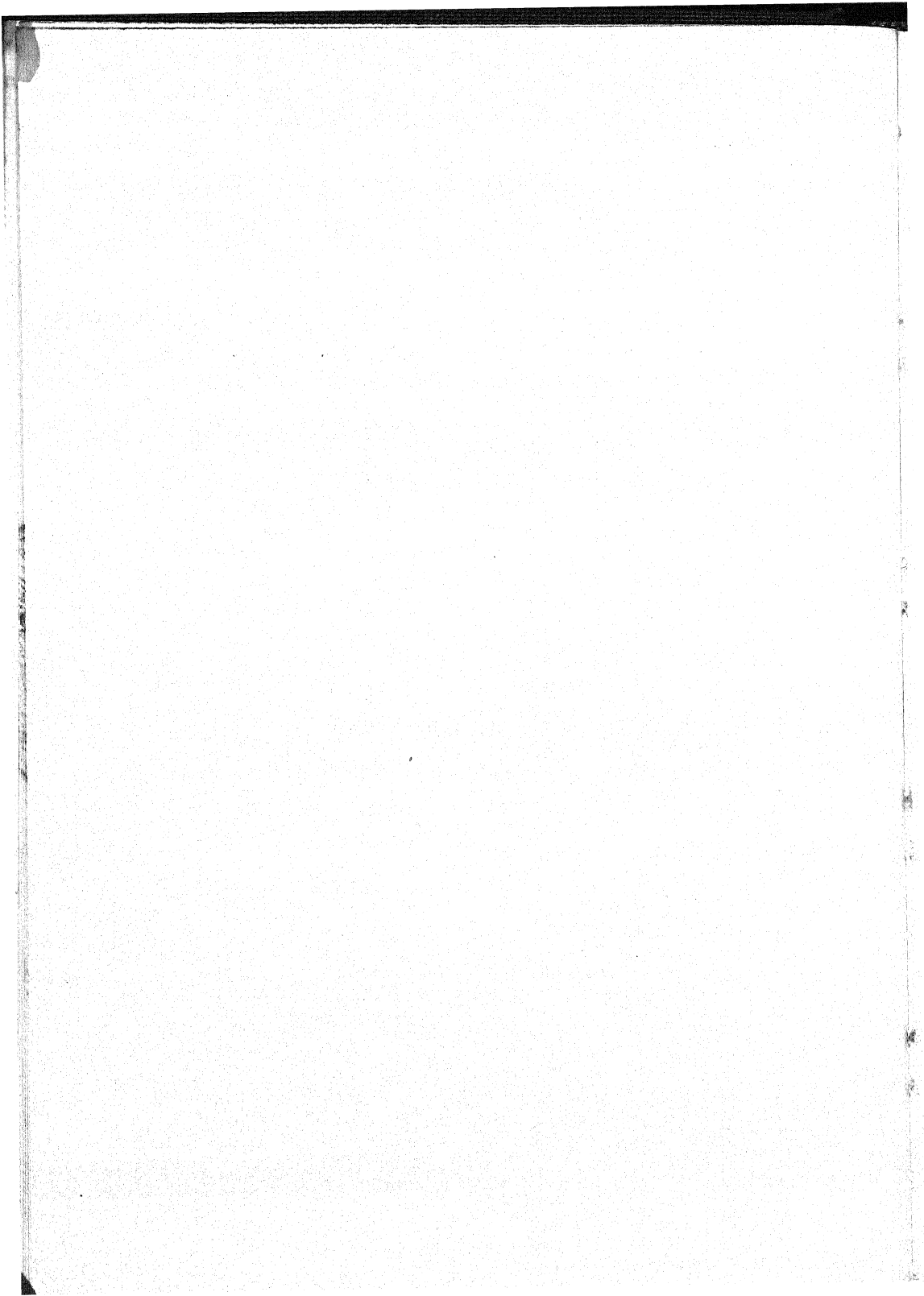
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# Spread Monolayers of Protein

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It has been more than forty years since Devaux (1) first studied the physical properties of films of proteins spread on water. There were, however, few papers on such films until Gorter began his investigations in the nineteen twenties. Interest in spread protein films appears to have reached a high point in the late nineteen thirties. Coinciding with the war years there has been a considerable decline in the number of papers on spread films of proteins, and the present, therefore, is an appropriate time to summarize and to consolidate our position.

Spread monolayers of proteins have been previously reviewed by Gorter (2) and by Neurath and Bull (3) and by Bateman (4).

## I. TECHNIQUE OF SPREADING

There are two methods of effecting spreading of proteins on aqueous surfaces. Hughes and Rideal (5), Hughes (6), Fosbinder and Lessig (7) found that solid particles, even of water insoluble proteins, spread very rapidly when carefully placed on the surface. The amount of material spread is determined by a modified Nernst balance. The other method which has several advantages over the solid particle method is to drop on the clean aqueous surface a dilute solution of the protein. It is very important that the protein solution be quite dilute but it is difficult to set

the upper limit for the concentration because the completeness of spreading is influenced by a number of factors. These factors are: The concentration of the spreading solution, the total amount of protein added as compared with the surface available for spreading, the composition of the substrate, the nature of the protein being spread as well as the time allowed for spreading. It has been the experience of the reviewer that if the concentration of the protein solution exceeds about 0.05%, spreading even under the most favorable conditions is apt to be incomplete. Mitchell (8), working with gliadin, found that at 0.1% protein concentration the spreading was incomplete while 0.01% gave complete spreading. Stållberg (9) reports that serum albumin when dissolved in a 60% solution of normal or isopropyl alcohol to which a small amount of sodium acetate had been added serves as an excellent spreading solution.

In each individual case, the spread areas per unit weight of protein must be demonstrated to be independent of the protein concentration, in the concentration range which is employed. This is certainly a necessary precaution before the serious study of the surface films of any protein is undertaken.

The actual method of placing the dilute solution of protein on the surface appears to be relatively unimportant provided the conditions are favorable for spreading. Common sense dictates that the drop size be small and that the drops be distributed over the available surface and not confined to a small area. The drops of protein solution are touched to the surface rather than allowed to fall on the surface.

Probably the most important single improvement in spreading technique has been the development of the Blodgett pipette (10) which allows a convenient and accurate handling of small volumes of spreading solution. The accuracy of delivery of such a pipette is indeed astonishing (see Fig. 1).

The choice of the substrate solution upon which spreading is done has to be governed by the needs of the experiment. In general, a protein will spread quite well on a dilute buffer solution which has a pH close to the isoelectric point of the protein. Good spreading is also obtained on substrates of high acidity such as 0.01 *N* to 0.10 *N* HCl and also on substrates of relatively high alkalinity. A plot of the extent of spreading, as measured by Gorter's so-called limiting areas, against the pH of the substrate solution yields a W-shaped curve, the maximum area coinciding with the isoelectric point of the protein. The prongs of the W correspond to strong acid and strong base substrate solutions. Langmuir (11) has measured the thickness of protein film deposited on chromium plated metal slides at various pH values of the substrate solution. He finds the thickness of such films to be independent of the extent of

spreading. The conclusion is, therefore, reached that the failure of a protein to spread at intermediate pH values is due to the loss of the protein in the interior of the substrate solution. This is not surprising. Native proteins are for the most part very soluble in water, and if the protein molecules carry a net electrical charge, there is a considerable energy barrier to overcome as they move from the water phase to the surface. Strong acid or strong base tends to diminish the effect of the electrical charge on the spreading.

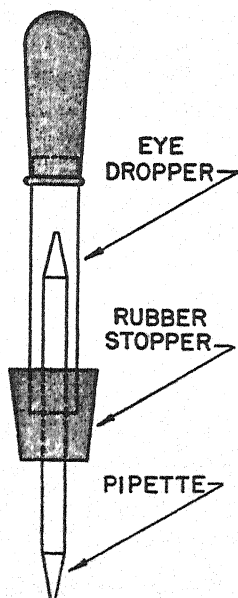


Fig. 1. Blodgett pipette used to deliver small volumes of solution.

Failure to spread may also result if the area of the substrate solution is too small or if the protein is made to spread against a film pressure. This occurs even at the isoelectric point of the protein. Joly (12) distinguishes two types of films. The A-films of proteins are those films which are completely spread on a large area against zero film pressure. These films are of great interest because their character is much better defined than that of the so-called B-films. The B-films result when the protein is confined to a small area and has to expand against a film pressure. B-films are very viscous and they may be more than one molecule thick.

It was reported (13) that as the salt concentration of the substrate solution was increased, the ease of spreading greatly increased. Sea-

stone (14) found that substrate solution of concentrated ammonium sulfate greatly facilitated protein spreading. Bull (15) confirmed this observation and has found that at least for egg albumin and for  $\beta$ -lactoglobulin, substrate solutions of ammonium sulfate from 10 to 35% are ideal and it is almost impossible not to obtain good spreading of these proteins on such substrate solutions.

It is most important to have the substrate solution free of surface active impurities. These impurities can be removed by treating the substrate solution with activated charcoal and then filtering the charcoal off. It is also important to have the substrate solution free of heavy metal cations.

The time required for complete spreading after the protein has been placed on the surface is a mooted question. Mitchell (8) recommends that the protein films remain on the substrate surface for 24 hours before measurements on the films be started. Cockbain and Schulman (16) allow two to five hours for complete spreading. Jonxis (17) working with different hemoglobins reports that complete spreading was obtained within 35 minutes. Certainly, if conditions are favorable for spreading and no protein escapes into the substrate solution, spreading is a remarkably rapid process. Egg albumin and  $\beta$ -lactoglobulin films when spread on concentrated ammonium sulfate solutions can be compressed within one minute after spreading and the same area of the film will be observed as when the films are compressed after several hours. In the experience of the reviewer if complete spreading is not obtained within fifteen minutes after spreading, there is an imperfection in the spreading technique which must be corrected before reliable results can be obtained.

The substrate solution is placed in a shallow tray. The surface area of the substrate solution should be as large as is convenient and some accurate method of registering the area occupied by the spread film must be available. Attention must be paid to the possibility of contamination by heavy metal cations. Probably the best material for a tray is glass (18) although the reviewer has found the cast aluminum tray sold by Central Scientific Company satisfactory provided the chromium plated brass plug is removed and replaced by a non-metallic plug. Before use the tray is paraffined by painting the hot dry tray with a hot solution of a good grade of clear hard paraffin dissolved in benzene. The tray is cooled and washed exhaustively with cold running water. Langmuir (11) recommends that the tray be coated with ferric stearate.

The tray along with whatever auxiliary apparatus is being used should be enclosed in a cabinet to decrease the accidental surface contamination by dust and grease from the air.

After the protein film is spread it can be subjected to a number of physical measurements. The most important of these measurements is the determination of the film pressure as a function of the film area.

## II. FILM PRESSURE DETERMINATIONS

The original technique employed by Langmuir (19) to measure the film pressure was to have a movable float separating an area of clean surface from the surface film. The pressure was then measured by determining the counter weights necessary to maintain the float in its initial position. The surface pressure exerted on the film is thus equal to the difference in surface tension between the clean surface and that upon which the film is spread. This type of surface balance has been improved by Adam (20), by Harkins (21) and by Gorter (22). The Central Scientific Company sells a surface balance under the trade name of the Cenco hydrophil balance which yields satisfactory results provided not too high accuracy is required.

There are several objections to the float type of balance. One of the most serious is that as the film is compressed by the movable barrier, the film is apt to leak around the movable float. There are flexible barriers connecting the float with the sides of the tray and which are supposed to prevent such leakage, but the question of leakage usually presents a source of annoyance. Another difficulty is that it is hard to make the float type of balance sufficiently sensitive to low pressures and such balances have to be constructed by an expert machinist and are quite expensive.

The Guastalla (23) float balance, however, appears to be quite simple and easily constructed. Great sensitivity is claimed for it. It consists of a float suspended by threads. The float acts as a pendulum and the displacement of the pendulum from its vertical position is proportional to the force exerted by the film. The film is restrained by a waxed thread laid across the tray on the surface of the substrate solution and resting against the pendulum. The Guastalla balance has not been extensively used.

The Wilhelmy (24) balance has found wide favor in recent years. It consists simply of a vertical plate partially immersed in the substrate solution and suspended from an arm of an analytical balance. The surface tension exerts a downward pull on the plate. The pull in grams, when multiplied by the acceleration of gravity and divided by the total length of the interface between the solution and the plate, gives, after the appropriate buoyancy correction has been made, the surface tension in dynes per centimeter. The difference in surface tension between that of the clean surface and that after the film is spread is the film pressure.

This difference is, of course, independent of the buoyancy correction and, accordingly, when used in this way the buoyancy correction is unnecessary. One absolute requirement of the Wilhelmy method is that the substrate solution must wet the slide, *i.e.*, the angle of contact between the slide and solution must be zero. The Wilhelmy method has been employed by Dervichian (25), by Abribat and Dognon (26), by Harkins and Anderson (27) and by others. The reviewer has found thin microscope cover glasses which are  $6 \times 6$  cm. serve as excellent Wilhelmy plates. A battery of these plates fixed in a parallel position and properly spaced from each other can be suspended from an arm of a chain-o-matic balance and rather extreme sensitivity can be achieved. With the Wilhelmy balance there is no question of leakage around barriers as the spread film is compressed.

While we have, in accord with established convention, used the term "surface pressure," it must be realized that what we really mean is the difference in tension between that of the clean surface and that of the surface in the presence of the spread film. "Surface pressure" has the dimensions of a tension and not of a pressure.

### III. FORCE-AREA CURVES

As the spread film of protein is compressed with a movable barrier, the film pressure increases and the plot of the film pressure against the area of the spread film yields a rather characteristic type of curve. A large number of proteins have been examined in this way and the pressure-area curves are at first sight discouragingly monotonous. There is first a region at low pressures where the pressure increases rather slowly with decreasing area. This region grades gradually into a region in which the pressure increases much more rapidly with decreasing area. The transition between these regions usually occurs between one and five dynes per centimeter film pressure. After something approaching a linear relation between film pressure and film area with a fairly large negative slope is attained, an inflection is reached at about 20 dynes per centimeter. After this inflection the pressure-area curve has a smaller negative slope. There are no phase transitions such as are found with many other types of spread films (see Fig. 2). This characteristic pressure-area curve will now be rather arbitrarily divided for purposes of discussion into the region below one dyne per centimeter and called the low pressure region and into the region above one dyne per centimeter and called the high pressure region.

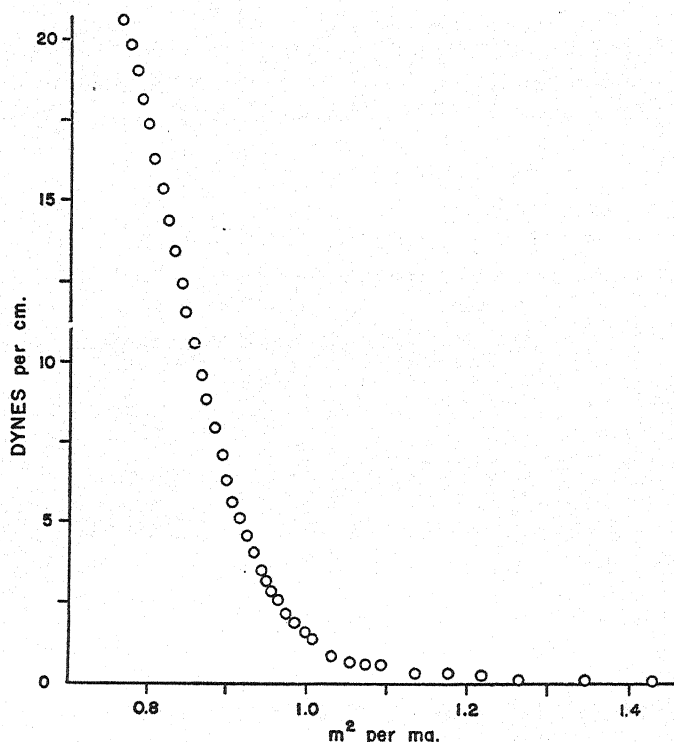


Fig. 2. Force-area curve of egg albumin spread on 35% ammonium sulfate.

#### IV. LOW PRESSURE REGION

The spread protein films in this region below one dyne per centimeter pressure have all the ear marks of a gaseous film. The reviewer has found for egg albumin, for  $\beta$ -lactoglobulin and for zein that the film pressure is a continuous inverse function of the area of the film, no matter how large the area of the film may be. Furthermore, when the film area is multiplied by the film pressure and plotted against the film pressure, a linear relation is obtained. Probably, many other protein films are gaseous in the low pressure region. Apparently, Guastalla (28) was the first to realize that spread protein films can be gaseous in the low pressure region and to calculate the molecular weights of the protein molecules in such spread films. He studied films of hemoglobin, of gliadin and of egg albumin.

The formation of gaseous protein films at the low pressures has been denied by Philippi (29), who reported that serum albumin and  $\beta$ -lactoglobulin exhibit a region from zero to 0.3 dyne per centimeter where



the film pressure is independent of the film area. Hughes and Rideal (5) claim that gliadin behaves in a similar manner. Philippi also reports that egg albumin and pepsin do not spread over the entire surface but form rigid coherent films. The findings of Guastalla and of the reviewer are in direct contradiction to the results of Philippi and of Hughes and Rideal. It is possible that the difference in spreading technique may account for these divergences. It is quite true that if the protein is added only to one end of a long tray that it takes some time for the protein to distribute itself evenly over the surface of the tray. Apparently, the rate of surface diffusion at high dilution of the film is quite small and one has to be careful to have uniform concentration over the surface just as one must be careful to have uniform concentration in a bulk solution before some property of the solution is measured.

A two-dimensional gas differs from a three-dimensional one in having two degrees of freedom instead of three. It is easy to show (30, 31), from kinetic theory that a gaseous film which obeys the ideal gas laws in two dimensions will follow the equation

$$FA = nRT \quad (1)$$

where  $R$  is the gas constant and is equal to  $8.31 \times 10^7$  ergs per degree per mole,  $n$  is the number of moles of gas,  $T$  is the absolute temperature,  $F$  is the surface pressure in dynes per centimeter and  $A$  is the area of the film in square centimeters.  $n$  is evidently equal to  $W/M$  where  $W$  is the weight of the film in grams and  $M$  is the molecular weight of the film molecules. Expressing the area,  $A$ , in square meters per milligram of protein, we have at  $25^\circ\text{C}$ . that  $M$ , the molecular weight, is equal to  $24.6 \times 10^7 / FA$ .

No actual surface film obeys the ideal gas laws except at infinite dilution, i.e., at very large areas. In general, corrections have to be applied both for the attractive forces between the surface molecules as well as for the fraction of the area occupied by them. One can write in formal analogy to Van der Waals' equation for real gases in bulk, the equation for a surface gas

$$(F + a/A^2)(A - b) = RT \quad (2)$$

where  $a$  is a constant involving the attractive forces between molecules,  $b$  is a constant which is proportional to the area occupied by the gas molecules and  $R$  is the gas constant. It has indeed been shown (20) that a number of films give a minimum in the curve when  $FA$  is plotted against  $F$  in the same way that  $PV$  when plotted against  $P$  for many actual gases exhibit a minimum point in the curve. A Van der Waals surface equation is therefore necessary to describe the behavior of such spread films.

Actually, egg albumin and  $\beta$ -lactoglobulin films do not give a

minimum when  $FA$  is plotted against  $F$ , although zein films appear to have a suggestion of such a minimum at 0.1 to 0.2 dyne per centimeter. This minimum in the case of zein may, however, be due to the displacement of small and soluble fragments from the film as the film is compressed. The absence of the minimum when  $FA$  is plotted against  $F$  means that the attractive forces between the surface molecules are negligible.

For those spread films which exhibit a strictly linear relation between  $FA$  and  $F$ , the equation is

$$FA = \alpha F + \beta \quad (3)$$

where  $\alpha$  is the slope of the straight line and  $\beta$  is the intercept of this line on the  $FA$ -axis. At zero pressure  $\beta$  is evidently equal to the limiting value of  $FA$  under conditions where the ideal gas laws are obeyed and, accordingly, the molecular weight of the film molecules is  $24.6 \times 10^2/\beta$ . This allows the calculation of the molecular weight of surface films from the value of  $\beta$ .

Dividing equation 3 by  $F$ , we see that at very large film pressures, the area of the gaseous film is equal to  $\alpha$  which allows us to calculate the area occupied by the gaseous molecules in their uncompressed condition.

Guastalla plots  $C/F$  against  $C$ , where  $C$  is the surface concentration in milligrams of protein per square meter of surface. He then extrapolates  $C/F$  to zero  $C$  to calculate the molecular weight of the film from the ideal gas laws. At 25°C. the molecular weight is  $24.6 \times 10^3 C/F$ . The Guastalla plot requires very accurate measurements of the film pressure at exceedingly low film pressures. For example, all of Guastalla's determinations for the three films which he studied were below 0.035 dyne per centimeter and extend down to 0.001 dyne per centimeter. This is certainly a great deal better than the reviewer has ever been able to do with his Wilhelmy balance, and the plot of  $C/F$  against  $C$  even for his best data is most unsatisfactory. The reason for the advantage of the plot of  $FA$  against  $F$  and the extrapolation to zero force over the Guastalla plot is quite evident. It is possible to obtain a great many experimental points from  $F = 0.10$  to  $F = 1.0$  dyne per centimeter and when  $FA$  is plotted against  $F$  a good straight line is obtained which can be extrapolated to  $F = 0$  with considerable confidence. In the case of gaseous egg albumin films at 25°,  $C$  is about 0.7 mg./meter<sup>2</sup> at a pressure of 0.1 dyne while at 1.0 dyne  $C$  is 0.83 mg./meter<sup>2</sup>. The plot of  $C/F$  against  $C$  thus embraces a concentration range of only 0.13 mg./meter<sup>2</sup> while the extrapolation has to be made from 0.7 mg./meter<sup>2</sup> to zero concentration. The values of Guastalla for the molecular weights

of the proteins which he studied are certainly reasonable, but he does not show his plots of  $C/F$  against  $C$  and, accordingly, it is not possible to judge how successful such plots actually were. The few investigations on gaseous films of proteins are summarized in Table I.

TABLE I  
*Some Properties of Gaseous Films of Proteins*

Protein	Molecular Wt. in film	Area in $M^2/mg.$	Substrate solution	Reference
Egg albumin	40,000	—	0.01 $N$ HCl	28
Egg albumin	44,000	0.97	35% $(NH_4)_2SO_4$	15
Gliadin	27,000	—	0.01 $N$ HCl	28
Hemoglobin	12,000	—	0.01 $N$ HCl	28
$\beta$ -Lactoglobulin	17,100	1.25	20% $(NH_4)_2SO_4$	32
$\beta$ -Lactoglobulin	34,300	1.40	20% $(NH_4)_2SO_4 + 2.5 \times 10^{-4} M$ $CuSO_4$	32
Zein	20,100	0.83	2% $(NH_4)_2SO_4$	32

The film molecular weight of egg albumin is close to the accepted value for this protein (33). The film molecular weight of  $\beta$ -lactoglobulin is half of that obtained by osmotic pressure measurements. It is concluded therefore that in the absence of  $Cu^{++}$  ions this protein dissociates on the surface. It was previously reported (34) that the film molecular weight of  $\beta$ -lactoglobulin was about 44,000. This value was obtained on samples of this protein which were prepared by a method which is now believed to lead to aggregation of the protein. The molecular weight of zein has not been established with certainty, however, Svedberg and Pedersen (35) report a value of 40,000. Zein, therefore, probably dissociates on the surface. The molecular weight of gliadin is about 27,000 which is in excellent agreement with Guastalla's value. It would appear that hemoglobin has undergone extensive dissociation at the surface since its bulk molecular weight is about 65,000.

The areas of the gaseous films are fairly small and are not very much larger than the areas of highly compressed films. This, as will be pointed out later, probably means that the peptide chains do not spread completely apart on the surface and, accordingly, the amino acid side chains maintain considerable vertical orientation even in the uncompressed film. The values of  $\alpha$  do not change appreciably as the film remains on the surface in the uncompressed state, so that there is little or no progressive expansion of the gaseous molecules.

While the term "gaseous" has been used to describe the protein films in the low pressure region, this term is undoubtedly a misnomer and has

been retained only because its usage has become established in the literature. The surface film undoubtedly exists in the form of a surface solution. In a truly gaseous film there would be no frictional forces acting on the protein molecules other than those resulting from the collision of neighboring protein molecules. Actually, the "gaseous" protein molecules certainly experience frictional forces from the water molecules in the surfaces as well as below the spread molecules.

#### V. HIGH PRESSURE REGION

An uncompressed spread film of protein can be freely blown about on the surface by a gentle current of air. This is evidenced by the fact that when talc is sprinkled on such an uncompressed film, the talc particles will readily respond to a gentle air current and after the air pressure is stopped the talc particles continue to move due to their momentum. Such a protein film as pointed out in the previous section is gaseous. As the film pressure is increased on the spread film the talc moves with less freedom, until finally a pressure is reached at which the spread film becomes fairly rigid, as evidenced by the fact that if the talc is displaced a small distance on the surface, it will return to its former position due to the elastic structure of the film. The film is said to have gelled. A protein film undergoes no true phase changes but is gradually transformed with increasing film pressure from a gas at large areas and small pressures to a gelled film at small areas and high pressures. It is the same kind of change experienced when a dilute protein solution in bulk is gradually concentrated until the solution becomes very viscous and finally gels.

The pressure region extending from about one dyne up to about 20 dynes pressure has been studied repeatedly and many force-area curves for this pressure range have been reported. The similarities between these curves are much more striking than their differences. The resemblance is not to be wondered at; this is the region in which the spread protein molecules are packed together. Since all proteins are made up of amino acids connected together by peptide bonds, and since the average molecular weights of these amino acids show no large variations from protein to protein, it is to be expected that the amino acid residues of the various proteins would pack at high film pressures to substantially the same area.

Gorter began the practice of characterizing the area occupied by a compressed film by what he called the "limiting area." This area is obtained by extrapolating the approximately linear portion of the force-area curve to zero pressure. The area found by this extrapolation is usually close to one square meter per milligram of protein. While the

Gorter method does offer a convenient basis for comparison of the spread films, three objections can be raised against it. First, the force-area curves are only approximately linear, and the more carefully the measurements are made the more evident does this departure from linearity become. Accordingly, the extrapolation of the "linear" portion of the force-area curve is ambiguous. Second, the areas obtained by such an extrapolation are too large (36) as is shown by X-ray diffraction measurements on proteins in the fiber form. Third, there is no physical justification for such an extrapolation.

Bull (37) and also Neurath and Bull (3) suggested that the coefficient of compressibility be used to characterize protein films in the high pressure region. Bateman and Chambers (38) have urged that the coefficient of elasticity be used for this purpose. The coefficient of elasticity is the reciprocal of the coefficient of compressibility so that these two suggestions are more or less equivalent and the same kind of information can be obtained from either. Since ordinarily the film is compressed rather than extended, the logic of using the coefficient of compressibility to characterize spread films is slightly more compelling.

The coefficient of compressibility of a spread film is given by

$$\delta = - \frac{dA}{AdF} \quad (4)$$

where  $A$  is expressed in square meters per milligram and  $F$  is in dynes per centimeter. When the coefficient of compressibility is plotted against the area of the film a well defined minimum is usually observed in this plot which allows the area and pressure at minimum compressibility to be assigned without ambiguity (see Fig. 3). It seems plausible to believe that the minimum compressibility of a protein film corresponds to the smallest area to which a film can be compressed without partial collapse of the film occurring. There is no proof that this is true but the following facts are strongly suggestive. The value calculated for the average cross sectional area per amino acid residue at the point of minimum compressibility is, in the case of egg albumin, in good agreement with X-ray diffraction studies on fibers of this protein (15). The sharp increase in the coefficient of compressibility after the critical area has been passed must indicate some rather fundamental change in the spread film at this point. Also it is noted that as the film is compressed to areas smaller than the critical area, the film pressures become much more uncertain and are much more dependent on time. This effect is particularly conspicuous when the film is compressed on a substrate solution of concentrated electrolyte such as 20%  $(\text{NH}_4)_2\text{SO}_4$ .

Zocher and Stiebel (39) devised a dark field ultramicroscope with

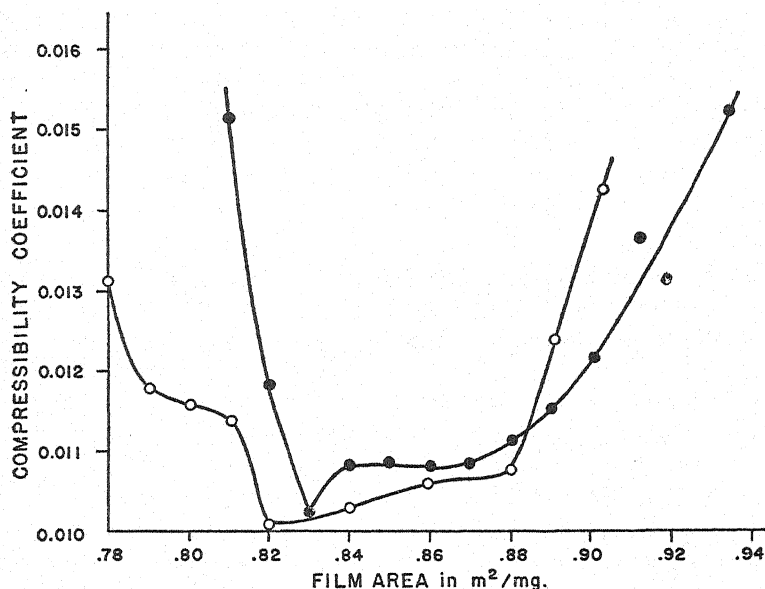


FIG. 3. Compressibility curves for egg albumin (full circles) and for  $\beta$ -lactoglobulin (half circles) films spread on 35 per cent ammonium sulfate.

which a spread film can be observed as it is compressed. This dark field microscope has been modified by Adam (40). Inhomogeneities in the spread film are easily detected by this method, since light is not scattered by a homogeneous monolayer, while any unspread material or aggregates formed incidental to collapsing of the film show up as brilliantly illuminated regions. Hughes and Rideal (5) found films of gliadin, glutenin and egg albumin exhibited pronounced collapse patterns which appeared as long folds perpendicular to the direction of compression. Similar patterns were reported by Neurath (41) with serum albumin at pH 4.8 and at film pressures greater than 14 dynes. Mitchell (8) reports that the earliest evidence of collapse appears to be a slight brightening of the field. Longitudinal folds parallel to the edges of the compressing slide do not occur very frequently with the proteins studied and are seen only at areas very considerably smaller than those corresponding to the onset of collapse. He reports the following collapse pressures for the proteins studied: Gliadin 14.1 dynes, zein 17.7 dynes, insulin 7-10 dynes. The appearance of collapse patterns certainly coincides rather closely with the film pressure at which the coefficient of compressibility is a minimum. Any studies on protein films above the collapse pressure must be qualified by the realization that such films have undergone deep seated changes.

It has been long recognized that the compression of a spread film of protein in the high pressure region involves the element of time. That is, as the film area is decreased by definite decrements the film pressure rises sharply and then slowly falls to a limiting value with time; the smaller the area of the film the longer is the time required for the pressure to reach a limiting value. Fourn and Schmitt (42) have studied this problem in some detail. The influence of time can be circumvented with a little patience; and the fact that spread films of proteins do exhibit this time effect is no valid objection to force-area measurements in the high pressure region, providing the compression of the film has been done slowly enough.

Above about 5 dynes, a protein film will almost always show some degree of hysteresis between the compression and the expansion curves, the expansion curve lying below the compression curve when film pressures are plotted against film areas.

It is evident from the force-area curves published in the literature that the films from some proteins are more compressible than others. These differences in compressibility in the high pressure region must be a reflection of the individual chemical differences in the various proteins. Langmuir and Waugh (43) have classified the amino acid residue side chains into three classes depending on the hydrophobic character of the residues. Class one includes those acids which have a hydrophobicity not exceeding that of one  $\text{CH}_2$  group. It was assumed by these workers that a hydrophilic group such as  $-\text{OH}$ ,  $-\text{NH}_2$  or  $-\text{COOH}$  neutralizes the effect of a neighboring  $\text{CH}_2$  or  $\text{CH}_3$ . A phenyl group was taken as equivalent to 3  $\text{CH}_2$  groups. Class 2 are those amino acids which have a hydrophobicity not exceeding 2  $\text{CH}_2$  groups while class 3 contain those acids whose hydrophobicity was equivalent to 3 or more  $\text{CH}_2$  groups.

Langmuir and Waugh considered that as the film pressure is increased, the most hydrophilic amino acids, *i.e.*, those of class one, are forced into the under phase and then as the pressure is further increased side chains of class 2 are displaced from the film and finally those of class 3.

To compare theory with experiment Langmuir and Waugh first compressed the protein films which they were studying to 30 dynes pressure and then expanded the films to one dyne pressure. These films were then recompressed and the area at one dyne was assigned the value of unity. By this technique they hoped to avoid difficulties arising from incompleteness of spreading. They then compared the areas of the films of gelatin, of gliadin, of edestin, of zein, of egg albumin and of insulin at 3 dynes and at 25 dynes with the areas at one dyne. They found a remarkable correspondence between these areas and the chemical composition of the proteins as given by their three hydrophobic classes of

amino acids. In fact, the compressibilities of the protein films at 3 dynes and at 25 dynes could be accurately predicted from the chemical composition of the proteins.

It is quite difficult to evaluate the findings of Langmuir and Waugh. In the first place their films were undoubtedly very incompletely spread because the concentration of protein in their spreading solutions was far too high; they used 0.333% protein solutions and the maximum concentration which can be used is about 0.05%. The areas they report finding are much smaller than those given by other workers. Compression of a protein film to 30 dynes pressure would undoubtedly lead to extensive collapse of the film. That such high pressures produced a collapsed film has been pointed out by Neurath (44). The so-called well-aged films of Langmuir and Waugh are simply well collapsed films.

While the reviewer is prepared to admit the correspondence between the compressibility of a spread film of a protein and the chemical composition of the protein as expressed by the hydrophobic and hydrophilic character of the amino acid residues he feels that the correspondence is due to at least two causes: First, a dehydration of the film as it is compressed and second, an orientation of the amino acid residue side chains. The relative importance of these two factors is a legitimate subject of controversy. Both factors should be very dependent on the hydrophobic and hydrophilic nature of the amino acid residues. There is, however, no evidence that at pressures below the collapse pressure any groups are forced out of the film in the sense of Langmuir and Waugh.

Proteins vary considerably in their affinity for water (45). It would be anticipated that films of the more hydrophilic proteins would be more compressible. The actual quantitative relation between the chemical composition of the proteins and their film compressibilities is, however, still obscure.

## VI. RESULTS OF FILM COMPRESSION STUDIES

An examination of the literature reveals that while pressure-area measurements have been made on a large number of protein films, in many cases very few actual data are reported. Even in the most favorable cases the authors have limited themselves to small graphs of the pressure-area curves. The reviewer has attempted to evaluate the film pressure in dynes per centimeter at minimum compressibility ( $F_m$ ), the area in square meters per milligram at the point of minimum compressibility ( $A_m$ ) and the minimum compressibility ( $\delta_m$ ) for as many films as appeared practical. The results of these calculations are shown in Table II.



TABLE II  
*Constants of Some Compressed Protein Films*

Protein	Substrate	$A_m$ M <sup>2</sup> /mg.	$F_m$ dynes/cm.	$\delta m$	Reference
Muscle hemoglobin	HCl pH 1	0.82	15	0.031	17
Horse hemoglobin	HCl pH 1	0.80	15	0.029	17
Horse hemoglobin	pH 6.8	0.75	18	0.015	17
Cow hemoglobin	pH 6.8	0.75	18	0.015	17
Oxidized cytochrome C	pH 10	0.78	10	0.043	46
Egg albumin	35% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.82	14.6	0.010	15
Egg albumin	N acetate				
	100 pH 4.6	0.71	15	0.028	42
$\beta$ -Lactoglobulin	35% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.83	16.7	0.010	34
Serum albumin	N acetate				
	20 pH 4.8	0.74	12	0.020	41
Zein	2% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	—	13	0.020	32
Palmitic acid (condensed film)				0.001	20

It would appear from Table II that the protein films pack to essentially the same area at the point of minimum compressibility, the average value for the 9 proteins in Table II being 0.78 M<sup>2</sup>/mg. The compressibility of the film, however, varies considerably and the same protein can show quite different values depending on the substrate. For example, the minimum compressibility of egg albumin film on a dilute acetate buffer is 0.028 while on 35% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> it is 0.010. Bateman and Chambers (38) investigated the elasticity of spread egg albumin films as a function of the pH of the substrate. In their first paper they concluded that the film elasticity was practically independent of pH, while in their second paper they report that the film elasticity exhibits a pronounced maximum at the isoelectric point. The influence of pH on the compressibility of spread films is obscure.

## VII. SURFACE POTENTIALS

Spread films can be characterized by surface potentials as well as by force-area measurements. In this type of measurement the change of the electrical potential difference between the substrate solution and air produced by the compression of the spread film is studied. The experimental arrangement generally used consists of a movable electrode above the surface of the film, a reversible electrode in the substrate solution and the proper external circuit which permits the measurement of the potential difference between the air and the substrate solution. Descrip-

tions of the apparatus have been given by Schulman and Rideal (47) Adam and Harding (48), Harkins (49), Philippi (29) and others.

There is another type of arrangement for the measurement of surface potentials. An insulated plate connected with an electrometer is held above the film; the plate and the substrate surface form a condenser whose capacity changes if the plate is moved in a direction vertical to the solution surface. If the plate be brought by means of an outside potential to the same potential as the substrate, no current flows when the plate is moved. This gives a means of measuring the potential at the surface. The horizontal plate may be made to vibrate by mechanical means and the resulting current flow used to locate equipotentials (50, 51).

It must be realized that the actual difference of potential between the air and substrate phase is of a far too complicated nature to be meaningful. The significant potential is the potential difference between the measured potential of the clean surface and that of the surface covered by the monolayer at some definite area or film pressure.

If the electrical double layer at the surface be treated as a plane plate condenser, we have

$$\Delta V = \frac{4\pi d\sigma}{D} \quad (5)$$

where  $\Delta V$  is the difference between the potential of the clean surface and the potential after the monolayer is spread,  $d$  is the thickness of the double layer,  $\sigma$  is the electrostatic charge per sq. cm. of surface and  $D$  is the dielectric constant in the double layer. If  $\mu$  is the average dipole moment of the molecules of the spread film and  $\theta$  be the angle of tilt of this dipole moment from a normal to the surface, then

$$\Delta V = \frac{4\pi n\mu \cos \theta}{D} \quad (6)$$

where  $n$  is now the number of molecules per sq. cm.

In general, as a protein film is compressed the potential difference  $\Delta V$  increases. Examination of equation 6 shows that this increase could be due to increasing number of molecules per unit area, to a decrease in  $\theta$ , i.e., to an orientation of the dipoles normal to the surface, or to a decrease of  $D$ . It is commonly assumed that  $D$  remains constant as the film is compressed (20). If  $D$  actually remains constant as the film is compressed, then the potential difference should be a function only of  $n$  and the angle of tilt.  $n$  can be calculated from the area of the film and any variation in  $\Delta V/n$  should be a function only of  $\cos \theta$ . For gliadin films, for serum albumin films, for  $\beta$ -lactoglobulin films and for

egg albumin films there is a region of low pressures, extending up to about one dyne per centimeter in which  $\Delta V/n$  is a constant. This indicates that in this region there is no change in orientation in the film as the film is compressed. At pressures above about one dyne per centimeter the slope of the potential-area curves ( $\Delta V/n$ ) decreases with increasing pressures. Now this is a very curious behavior because strictly interpreted this would mean that the angle of tilt to the normal ( $\theta$ ) must have increased with increasing pressures. This conclusion makes no sense at all and it is, therefore, felt that surface potential measurements cannot at the present time be described in terms of the simple theory at our disposal. One source of our confusion may be, as suggested to the writer by Dr. J. T. Edsall, that the axis of the dipole does not coincide with the long axis of the amino acid residues and when the residues are oriented vertically to the surface by increasing pressure, the dipole may be oriented in a different direction such as to decrease the electrical effects observed. Surface potential measurements have, however, considerable utility in the study of the interaction of molecules injected under a spread film and the molecules of the film. This interaction will be discussed presently.

#### VIII. PRESSURE DISPLACEMENT FROM A FILM

One is impressed by the very great insolubility of a completely spread protein film. As the film is compressed there is, above a certain pressure, a partial collapse of the film which is evidenced in the ultramicroscope as a brightening of the field. If the film is still further compressed, ridges visible to the naked eye appear in the film. There is, however, no evidence that any of the film protein has passed into solution in the substrate.

Langmuir and Waugh (52) have discussed the influence of film pressures on film solubility. According to Gibbs

$$dF/d \ln C = nRT \quad (7)$$

where  $C$  is the concentration of the substance in the underlying solution,  $n$  is the number of moles per sq. cm. and  $R$  is the gas constant. For a protein which occupies one sq. meter per milligram on the surface and has a molecular weight of 35,000,  $n$  would be  $2.85 \times 10^{-6}$ . Then at  $20^\circ\text{C}$ .

$$d \ln C = 220dF \quad (8)$$

Accordingly, as Langmuir and Waugh point out, an increase of film pressure of 15 dynes per centimeter should increase the solubility of the film by a factor of  $10^{33}$ . A spread film of protein is apparently extremely insoluble.

In their later publication Langmuir and Waugh studied the pressure soluble material produced by the peptic digestion of egg albumin and of insulin. They found both proteins suffered a loss of spread area as the digestion proceeded. However, both proteins were incompletely spread so that their results are hardly quantitative. The conclusion that digestion leads to pressure soluble fragments can, however, hardly be denied. They attempted to formulate a theory of pressure-solubility of peptide chains and to calculate the solubility of spread films as a function of the pressure and of the molecular weight. Their theory involves so many approximations as to be of little practical use. They conclude that polypeptide chains having the average composition of proteins should show pressure solubility in the range of 5 to 10 dynes per centimeter when the molecular weight is about 1,200, while if pressure solubility is observed between pressures of 20 and 25 dynes per centimeter, the molecular weight is about 1,700.

#### IX. PENETRATION OF PROTEIN FILMS

Closely related to the displacement of pressure soluble material from a spread film is the penetration of a film by surface active molecules injected under the film. Thus Schulman (53) reports that if the injected molecules have hydrophilic heads and hydrophobic tails three situations may arise: (1) If there is no association between the polar heads of the injected molecules and the polar groups in the film, no alteration in the film characteristic is noticed. (2) If there is association between the polar groups of the injected molecules and the film but no association between the hydrophobic tails and the film, there is an adsorption of the injected molecules under the film with consequent change in the surface potential but no increase in surface pressure. (3) If there is association between both the heads and tails of the injected molecules with the film, then a polar group of the film anchors a polar group of the injected molecule. The hydrophobic portion of the injected molecule associates with the hydrophobic portion of the film and thus penetration of the monolayer results. In this case the film pressure is greatly increased while the surface potential assumes a value which is an average one for the original monolayer and the injected molecules.

Film penetration can be completely prevented by compressing the film, prior to injection, to the equivalent pressure at which displacement of the penetrating substance would start. Film penetration, while of great interest, is very difficult to quantitate because of the unknown amount of the injected material which remains in solution in the substrate.

## X. MIXED FILMS

Schulman and Rideal (54) studied mixed films of gliadin and cholesterol. Taking advantage of the fact that gliadin and cholesterol are both soluble in ethyl alcohol, mixtures of these two substances were spread from an alcohol solution on an aqueous substrate. Stable mixed films were obtained up to a film pressure of 20 dynes while above 20 dynes the cholesterol-gliadin film suddenly liquefied and showed all the characteristics of a pure cholesterol film. It was found that this displacement of the gliadin was reversible as the pressure was lowered below 20 dynes.

The properties of mixed films of egg albumin and myristic acid, obtained by simultaneous spreading of these substances from solution, have been investigated by Neurath (55). By assuming that the protein occupies the same film area in the presence of the acid as it does when spread alone, no film interaction could be detected with films containing between 100 and 400 molecules of myristic acid per molecule of protein. With films containing less myristic acid, a marked increase in the area per fatty acid molecule was observed, and with a mole ratio of 9 reached a value of more than 180 sq. A. It is evident that myristic acid must at these molecular ratios have caused a considerable expansion of the egg albumin molecules on the surface.

Bull (56) spread mixtures of sodium lauryl sulfate and egg albumin on 35%  $(\text{NH}_4)_2\text{SO}_4$  solutions and found definite evidence for complex formation between the detergent and egg albumin. The sum of the spread areas of the detergent and of egg albumin separately was always greater than the area occupied by the mixture. It was assumed that the area of the egg albumin per unit weight was constant for a given film pressure and that the loss of area was due to an adsorption of the detergent on the film. On the basis of this assumption it was calculated that the first complex between egg albumin and detergent involves about 17 molecules of detergent per molecule of egg albumin while the second complex which forms at a higher ratio of detergent to protein contained about 34 such detergent molecules per protein molecule. The actual nature of these detergent-protein films is very obscure. Presumably such interaction as occurs takes place before spreading is accomplished, but where is the detergent located? There is no evidence that any component was displaced from the film when the film pressure was raised from 2.5 dynes to 15 dynes.

## XI. INDICATOR OILS AND EXPANSION PATTERNS

Blodgett (57) described the preparation of indicator oils which permit the outlining of a spread film on a surface. These oils are prepared quite simply by oxidizing automobile oil by prolonged heating and then

mixing this oxidized oil with pure mineral oil to give an oil with the desired spreading quality. When placed on a water surface these oils spread on the surface and show refraction colors and can thus be used to outline spread films of protein.

Schaefer (58) has studied the type of surface figures obtained when such indicator oils are placed on a compressed protein film. The actual technique employed to form the surface patterns was as follows: Indicator oil was placed on a clean water surface and a small amount of protein was then placed in the center of the oil film. The protein expands against the small pressure exerted by the oil and forms a more or less circular film in the center of the oil film. The oil film is brilliantly colored while the protein film is perfectly clear and without color. A small drop of indicator oil is then placed in the center of the protein film. The geometrical surface figure is then developed by the spreading of the drop of indicator oil. Schaefer distinguishes three types of geometrical figures. A star-like figure was shown by egg albumin, pepsin, pepsinogen, trypsinogen, urease, edestin, tobacco seed globulin and tobacco mosaic virus. A rough circular pattern was shown by trypsin, gliadin and papain. A smooth circular pattern was given by gliadin acetate, zein, casein, protamine, insulin, protamine-insulin and gelatin. While these figures appeared to be quite characteristic of a protein when spread on pure water (tobacco mosaic virus was spread on concentrated  $(\text{NH}_4)_2\text{SO}_4$  solutions), the various figures could be modified by denaturation previous to spreading, by the addition of  $\text{Cu}^{++}$  or  $\text{Zn}^{++}$  ions to the substrate and by the addition of acids or of bases to the substrate. Thus native pepsin gives a star-like figure but if the pepsin is denatured by ultraviolet irradiation, by heat or by shaking the pepsin solution, a smooth circular figure is obtained. The addition of  $\text{Cu}^{++}$  or  $\text{Zn}^{++}$  ions to the substrate changed the insulin figure from a smooth circle to a star-like one.

The production of surface patterns is undoubtedly a reflection of the structure in the protein film and amounts to a more elegant and spectacular method for the detection of film gelation. That is, those films which give a star-like pattern are undoubtedly gels while the smooth circular figures arise from films which are gaseous. The formation of a gel is due to molecular interaction in the spread film.

## XII. VISCOSITY OF FILMS

The viscosity of surface films has been measured in one of two ways. The passage of the film through a small channel can be observed (59, 60) or the oscillations of a disk in the surface can be measured (11, 61, 62, 63).

There are several points which emerge from these film viscosity studies. Langmuir (63) reports the film viscosities of 15 proteins at 2 dynes per centimeter and at 6 dynes per centimeter pressure. Some of these films were also subjected to pressures of 10, of 16.5, of 19 and of 29.5 dynes per centimeter. It is evident from Langmuir's results that the film viscosity increases greatly as the film pressures are increased and further that the various proteins at the same film pressure show tremendously different viscosities. For example, a gliadin film at a film pressure of 2 dynes per centimeter had a viscosity of 0.001 C.G.S. units while a horse globulin film at the same pressure had a viscosity of 120 C.G.S. units. Langmuir points out that those protein films which have a high viscosity give star-like surface patterns with indicator oils while those with low viscosities exhibit smooth circular patterns. This observation gives a very strong hint as to the principal factor involved in film viscosity. The high viscosities are caused by interaction of the polar groups of neighboring spread protein molecules. These same groups lead to gelation of the film. If the viscosities could be studied in the low pressure region where the films are gaseous and the interaction between spread molecules is negligible much useful information could be obtained regarding the degree of asymmetry of the spread molecules, in close analogy with viscosity studies on dilute solutions of proteins.

Probably some idea about the shape of spread protein molecules could also be obtained by studying the rate of surface diffusion at low pressures. To date no such investigation has appeared although it should be comparatively easy to devise an experimental procedure.

Joly (12) reports that the film viscosity is dependent on the mode of spreading. Proteins which are allowed to spread freely on a large surface area give rise to so-called A-films which are fluid and stable with a low viscosity. If the area of the spreading surface is limited and the protein has to expand against a pressure, another type of film is formed. These are the so-called B-films. These films are viscous and show the phenomena of age. It is probable that Langmuir's films resemble more closely B-films than they do A-films.

### XIII. REACTIONS IN MONOLAYERS

Due to the inherent technical difficulties, the chemical reactions of spread protein monolayers have not been extensively investigated. Schulman and Hughes (64) report that the injection of chymotrypsin under a casein film causes hydrolysis of the film as evidenced by a change in the film potential. They also report that pepsin injected under a casein film will digest the film at pH 2.

Rideal (65), Neurath (41) and Gorter (66) find that when a spread

protein film is irradiated with ultraviolet light, the film collapses. This collapse is presumably due to fragmentation of the protein molecule by the ultraviolet light with the production of soluble, pressure displaceable material.

#### XIV. STRUCTURE OF PROTEIN MONOLAYERS

It is generally agreed that proteins consist of amino acid residues connected together through peptide bonds to form peptide chains which may embrace several hundred such residues. The arrangement of the peptide chain or chains in the protein molecule is still obscure. Whatever the arrangement of the chain may be, the protein molecule can exhibit a high degree of internal regularity, as is revealed by X-ray diffraction studies (67). Such molecules amount to molecular crystals. It is one of the remarkable facts of nature that when such soluble, highly organized native protein molecules are placed on an aqueous surface that they promptly spread on the surface to form insoluble films whose thickness corresponds to one peptide chain irrespective of the dimensions of the original, native molecule.

It has been suggested that proteins exist on a surface in the form of straightened  $\beta$ -keratin chains and at low film pressures the amino acid residue side chains are lying flat on the surface and as the film is compressed, these side chains are oriented normal to the surface (3). The reviewer has come to doubt this theory for the following reasons: The average side chain spacing of the peptide chain of a protein is about 10 Å while the distance along the  $\beta$ -keratin chain per residue is about 3.3 Å (68). The area occupied by a residue lying flat on the surface would then be about 33 sq. Å. The average residue weight (69) of most proteins is around 115 and, accordingly, the area occupied by a peptide chain with the side chains lying flat on the surface would be

$$\frac{33 \times 6.02 \times 10^{23}}{115 \times 10^{23}}$$

or about 1.7 sq. meters per milligram of protein. Referring to Table I, we see that the area of the uncompressed protein film in the gaseous region is considerably less than 1.7 sq. meters per milligram of protein. The conclusion is therefore drawn that the amino acid residue side chains do not lie flat on the surface at low pressures but have a considerable degree of orientation normal to the surface.

It is commonly held that in a compressed state the side chain residues are oriented vertically to the surface. That this is indeed a valid interpretation is shown by the good agreement between the observed film areas (Table II) and the areas calculated on the basis of the known



average dimensions of the amino acid residues. The backbone spacing of a stretched peptide chain is about 4.65 Å while the distance along such a chain per residue is as we have seen 3.3 Å. Accordingly the average cross sectional area per residue is about 15.3 sq. Å. Then the area of the compressed film must be

$$\frac{15.3 \times 6.02 \times 10^{23}}{115 \times 10^{23}}$$

or about 0.80 sq. meters per milligram of protein. This value compares favorably with the results reported in Table II. Actually, in a  $\beta$ -keratin chain the side chain residues alternate above and below the main peptide chain so that the actual average area per residue is about 30.6 sq. Å instead of 15.3 sq. Å as given above. Naturally, if half the residues are above and half below the main peptide chain, the area occupied by the chain will be half of the sum of the area of all the residues. It is concluded from these calculations that the peptide chains exist on a surface with the side chain residues oriented normal to the surface, and further that approximately half of these residues are directed towards the water phase and the other half towards the air phase. While the degree of orientation is not as complete in an uncompressed film as it is in a compressed one, the compressed and uncompressed films have essentially the same structure.

If a scaled molecular model of a peptide chain be constructed with freedom of rotation about the valence bonds, it is of course possible for the model chain to assume a great variety of configurations. However, if the model peptide chain be placed on a plane surface and confined to this surface, it will be found that the chain has become very rigid and the only freedom allowed will be in the side chains beyond the  $\beta$ -carbon atom. Probably once a peptide chain has been spread on a water surface its structure is fixed and is, short of collapse of the film, independent of the degree of compression.

In order to spread on a water surface and form a stable film a substance has to have hydrophilic and hydrophobic groups in it. The bottom side of a spread film of protein must be predominantly hydrophilic, while the side directed towards the air must be predominantly hydrophobic, otherwise a protein monolayer would not be stable on the water surface. That the air side of a protein monolayer is predominantly hydrophobic is shown by the experiments of Bull (70) who deposited single monolayers of egg albumin at various pressures on glass slides and measured the contact angle between water and the deposited film. The film deposited at 15 dynes pressure showed an adhesion tension of about 100 ergs per sq. cm. The adhesion tension of a pure hydro-

carbon (paraffin) surface is about 50 ergs per sq. cm. while the tension on a surface which is completely wet by water is 144 ergs per sq. cm. It appears, therefore, that while the top side of a spread film is not exclusively hydrophobic, it does have a pronounced hydrophobic character.

Now whatever may be the manner of folding of the peptide chains either in the native or in the spread protein, it is not, in general, possible for consecutive amino acid residue side chains to be oriented in the same direction; there is not enough space along the chains to accommodate the side chains (71), and consecutive residues must be oriented in opposite directions. This means that there must be a strong tendency for hydrophobic and hydrophilic residues to occupy alternate positions along the peptide chain. The positions of the residues in the peptide chain cannot be changed once the peptide chain is formed and, accordingly, in the native as well as in the spread peptide chains every other residue will tend to have a hydrophilic group in it.

It has been proposed that native protein molecules are made up of parallel sheets or layers of peptide chains (72, 73, 74, 75). It is reasonable to believe that these layers have the same hydrophilic-hydrophobic characteristics as do spread films. Thus in a native molecule containing two such layers of peptide chains, the outer faces of the molecule would be predominantly hydrophilic with a hydrophobic sandwich in between. Dervichian, for example, has drawn an analogy between a soap micelle and a protein molecule as far as the arrangement of the hydrophilic and hydrophobic groups are concerned. Certainly, there is much evidence to substantiate such a picture. While most of this evidence is of an indirect nature, it is no less compelling. Without the layer structure of the native molecule as the guiding idea, the formation of protein spread monolayers is difficult to understand.

The actual process by which a layered native molecule is opened up and spread is obscure. There must be cleavage along the hydrophobic as well as along the hydrophilic planes. Sometimes this cleavage results in a dissociation of the protein molecule as is the case with  $\beta$ -lactoglobulin, hemoglobin and zein; while other molecules such as those of gliadin and egg albumin do not dissociate. Due to rigidity of the sheets of peptide chains the actual cleavage of a plane must proceed in one step and would involve the simultaneous separation of as many as 50 or more side chain residues in one face from a like number in the opposite face.

As emphasized by Dervichian, the only requirement of the native molecule to spread in a monolayer is that the native molecule must have at least two layers of peptide chains. How many layers of peptide chains the native molecule actually has is in most cases unknown. X-ray

diffraction analysis indicates that a methemoglobin molecule has four such planes (75). A two plane structure would by necessity lead to a rather asymmetric molecule. X-ray diffraction indicates (67) that insulin and  $\beta$ -lactoglobulin molecules are not pronouncedly asymmetric. What the true situation is, is impossible to say at the present time. In any event, the thickness of a protein molecule should be, if the layer structure is correct, some simple multiple of about 20 Å.

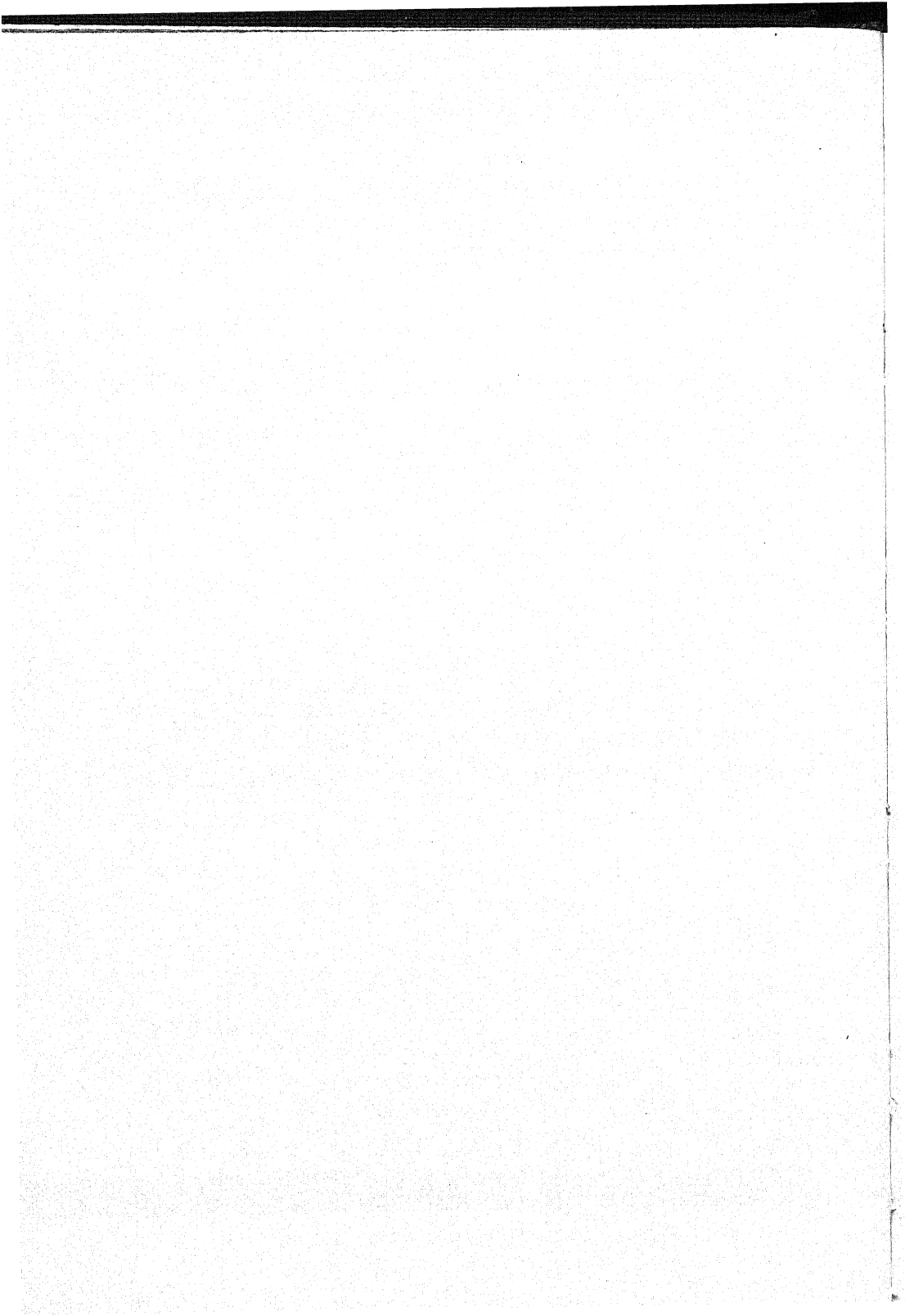
The nature of the folding of the spread protein as well as the sequence of the amino acid residues in the chain, two problems of great importance to protein chemistry, cannot at the present time be studied by spread film technique.

The interesting question of the specific biological properties of spread protein films and of deposited spread films is the subject of a separate review by A. Rothen which appears elsewhere in this volume.

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# Films of Protein in Biological Processes

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## I. INTRODUCTION

The importance of surface phenomena in biology has been recognized for a long time. This importance is due to the considerable extension of interphases in the animal body caused by the small size of the basic structural unit of living organisms, the cell. Though many of the chemical reactions which occur in a homogeneous phase can likewise occur at an interphase, it is to be expected that the interphase offers conditions which may alter reactions involving proteins.

Proteins have a very remarkable property in common, that of spreading easily at a water interphase. This spreading usually involves a considerable change in the shape of the protein molecule. The steric architecture of the molecule is therefore lost in the process, the molecule is unfolded, and when the process goes to completion, the thickness of the protein film is that of an average polypeptide chain. It is of great interest to learn how the biological activity of proteins is affected by this unleafing process occurring at interphases. The study of proteins at such interphases *in vitro* may give some clue to the processes involved *in vivo*.

Three general methods are used in testing films of proteins for biological activity:

(1) The tests are made at the water interphase where the films are spread.

(2) The films are collected at the interphase, suspended or dissolved in aqueous media, and subsequently assayed in animals.

(3) The films are transferred onto metal or glass slides which are convenient for studies *in vitro*.

The English workers in the field, mainly Rideal and his school, have extensively used the first method. The major part of their work deals with non-protein films and is therefore of no direct concern here. However, some of their findings do have a bearing on protein films. They demonstrated that if the molecules in the monolayers react with a substance dissolved in the substrate, the rate of reaction may be modified by a change in the orientation of the molecule of the layers brought about by compression. For instance (1), when, in a film of lecithin, the area occupied by a single molecule is  $0.96 \times 10^{-14}$  cm.<sup>2</sup> the half life of the molecule is 0.5 minute if the underlying solution contains 0.001 per cent black tiger snake venom, the half life increases to 90 minutes when, following compression, the area occupied by a single molecule is  $0.47 \times 10^{-16}$  cm.<sup>2</sup> They also demonstrated (2) that the orientation of long chain esters, at the oil-water interphase was a prime factor for the hydrolysis by pancreatin. Films of esters of acids with 2, 3 or 8 carbon atoms are digestible as contrasted to acid chains containing 5 or 6 carbon atoms. The explanation offered by the authors is that in the former type the orientation of the alkyl residues is in "cis" position, leaving the C-O grouping available to the enzyme contained in the substrate while the indigestible esters have the "trans" configuration in which the alkyl residues protect the C-O bond from the enzyme.

## II. TRANSFER OF FILMS

The transfer of films on slides in conjunction with the measurement of their thickness by optical means has been used extensively for testing biological activity. In 1934, K. B. Blodgett (3) demonstrated that films spread on an aqueous solution can easily be transferred to slides when the latter cross the interphase downwards or upwards. The condition required for the transfer is that sufficient surface pressure be applied to the film (from a few dynes up to 20 dynes, depending on the nature of the film) to force it on the slide. The shallow spreading trough is built with a deeper well at one end to allow the dipping of the slide to a sufficient depth. Convenient holders have been constructed to transfer a film on more than one slide at a time.

Blodgett and Langmuir (4) developed an optical method to determine the thickness of transferred films such as protein films. This method, now well known, has often been reviewed (5). It consists in depositing on a polished metal slide enough monolayers of a reference film, barium stearate for instance, to produce interference phenomena between a linearly polarized beam of light reflected from the air-film interphase

and the film-metal interphase. In order to obtain interferences the added thickness must be of the order of a quarter of the wavelength of the light. This "optical gauge" is built up of adjacent steps of multilayers of barium stearate in such a way that there is a difference of two layers in the thickness of the successive steps. The measurement depends on the determination of the angle of incidence at which two adjacent steps appear of the same intensity. When a film of protein of unknown thickness is deposited on the slide covered with the optical gauge the angle of incidence must be shifted to reestablish equal illumination of two adjacent steps. This shift in the angle of incidence is a measure of the thickness of the film. The accuracy of this simple method is somewhat lower than commonly assumed for two main reasons. First, depending on the thickness of the unknown film, two different pairs of steps before and after the deposition require matching, consequently small irregularities in the optical thickness of individual steps interfere with a precise measurement. Second, the change of the intensity of the light reflected by a step with the angle of incidence is relatively large only for angles greater than  $75^\circ$ . For smaller angles the matching of two steps cannot be accomplished accurately. Under the best conditions optical thicknesses can be determined within a few Angström units but discrepancies as high as 10 Å can easily occur. Finally, the method is time consuming when a large number of measurements is required since it necessitates for each measurement the building up of an optical gauge of some 50 odd layers of barium stearate.

Waugh (6) recently designed an apparatus to measure optical thicknesses based on the same principle. The apparatus is so constructed that the optical gauge is built on a different slide from the one on which the investigated specimen is deposited. The accuracy obtained is of the order of 10 Å.

The method of Blodgett and Langmuir is based on a special case of a more general phenomenon which comprises the properties of linearly polarized light reflected from a surface coated with films of thickness varying from a few Angström units to within the range of a  $\frac{1}{4}$  wavelength of light. Drude (7) devised, more than fifty years ago, a method based on this general phenomenon. He showed that films only a few Angström units thick, covering a metallic reflecting surface, could be measured by analyzing the change in ellipticity of the reflected light brought about by the film. Drude himself, analyzing the ellipticity with a Babinet compensator, demonstrated that films covering polished metallic surfaces are of molecular dimensions. Since then several authors have used this property to determine film thickness (8, 9). A simple instrument based on a modification of this method was recently



described (10). The original feature of this instrument consists in a new half-shadow arrangement to follow the change in ellipticity. The working principle is as follows: Linearly polarized light vibrating at  $45^\circ$  to the plane of incidence is reflected from a polished metal plate. The upper and lower parts of the plate have been coated with one and three monolayers of a reference film (barium stearate), respectively. Since the thicknesses of the films covering the upper and lower parts are different (24.4 and 73.2 Å, respectively) the ellipses representing the vibrations of the beams reflected from these two areas differ in their orientation and ellipticity. The two beams of light characterized by these ellipses go through a mica  $\lambda/4$  plate which is oriented with its "fast" direction parallel to the bisector of the angle made by the major axes of the ellipses. This angle is only  $1^\circ 56'$  in the case of a reference film of barium stearate. After passing through the  $\lambda/4$  plate the two beams of light are characterized by two extremely elongated ellipses with ellipticities of less than  $1^\circ$ . Their major axes make an angle of about  $3^\circ$ . Finally the light goes through an analyzing Nicol. Since the ellipticity of both ellipses is small, the angle made by the two major axes of the elongated ellipses corresponds to the "half-shadow" angle of a polarimeter. The observation is made through an ocular focussed on the plate. The angle read on the analyzer when both halves of the field corresponding to the upper and lower parts of the slide appear of the same intensity, gives the zero position of the apparatus. If now a thin unknown film is added on the whole reflecting surface, the upper and lower parts become unequal in intensity since the ellipses characteristic of the beams after reflection have rotated and have different ellipticity. Consequently the elongated ellipses obtained after the  $\lambda/4$  plate have also rotated. Turning the analyzer in the same direction brings back equal intensity of both halves of the field. The difference in the position of the analyzer before and after deposition of the film is a measure of its thickness which can thus be determined within  $\pm 0.3$  Å.

The measurement of film thickness by optical methods requires knowledge of the refractive index of the film. Since the refractive index of proteins falls within a narrow range, no serious error is made by assuming an average value.

*A priori*, the thickness of a protein film at the water interphase is not necessarily the same as that determined after transfer to the slide. However, in most cases tested the area covered by the film at the water interphase is close to the area it occupies on the slide after transfer. In the past, the thickness of protein films has often been determined indirectly by assuming that none of the spreading material had gone into solution. The determinations of molecular weights of proteins by force-

area diagrams have demonstrated that this is true if the necessary precautions are taken.

### III. SPREADING AND BIOLOGICAL ACTIVITY

In studying the biological properties of spread protein films one should always differentiate between films of unfolded molecules and films of adsorbed, unchanged or slightly changed molecules. Much confusion has arisen for lack of appreciation of this difference. Spreading at the water interphase does not necessarily mean that complete unfolding has occurred. Determination of the thickness of the film is the only criterion which indicates whether, and to what extent, the molecules have unfolded. It is to be expected that a film of adsorbed molecules would exhibit the activity of the material in solution. It might be said at this point that unfolding of proteins in spreading is a less complex phenomenon than surface denaturation. Perhaps spreading could be looked upon as the first step of surface denaturation, the second step involving crumpling of the film and subsequent aggregation.

### IV. FILMS OF ENZYMES

#### 1. *Pepsin and Trypsin*

Gorter (11) spread pepsin on a substrate at pH 2.85 and trypsin on a substrate at pH 7.00. He removed the films with a silk net and dissolved the pepsin film in an acid medium at pH 4 and the trypsin film at pH 5. He showed that the activity of both enzymes was at least 80 per cent that of the original. From these experiments it cannot be decided whether the film itself was active or whether the activity was regained during solution. Langmuir and Schaefer (12) transferred unfolded films of pepsin on metal slides and tested their films for milk-clotting activity. They reported some activity, but with better controlled experiments (13) they came to the conclusion that the activity was due not to the pepsin film but to the pepsin removed from the slide during the testing. According to the authors the activity was regained during the dissolution of the film. This would suggest that in Gorter's experiments the activity may also have been regained after the dissolution of the films.

The following experiments made with films of trypsin showed that inactivation had occurred on spreading (14). As will be seen later, spread films of antigens deposited on slides retain their ability to react specifically with homologous immune sera. However, a drop of trypsin solution at a pH above 7 destroys this specific property of the film. If a film of trypsin is first deposited on the slide and then the film of antigen, no inactivation of the antigen film occurs even when a drop of

buffer at  $\text{pH} > 7$  is placed on the slide since the film is still capable of reacting subsequently with homologous sera.

## 2. Urease

Langmuir and Schaefer (13) studied the activity of films of unfolded urease transferred on metal plates by following the rate of decomposition of urea. Their final conclusion was that only 5 per cent of the original activity was exhibited by the film. Furthermore, if the film was left standing at the water interphase for 15 minutes before transfer, only 2 per cent of the original activity remained. This points strongly towards complete inactivation of the urease by unfolding.

## 3. Catalase

Films of catalase were also investigated by Langmuir and Schaefer (13). They reported that films of catalase 23 Å thick deposited on metal slides decomposed a solution of hydrogen peroxide on immersion of the slides. The relatively large thickness of the films indicates that complete unfolding had probably not occurred.

The same enzyme was studied extensively by Harkins, Fourt and Fourt (15). However, most of their work was carried out with adsorbed enzyme. They adsorbed catalase molecules on plates conditioned with thorium nitrate. An increase in thickness of 55 Å followed the adsorption. The activity of the adsorbed catalase when tested in 0.008 *M* hydrogen peroxide proved to be 1/5 to 1/10 that of the original material. The same authors in addition carried out some immunological tests with alternate adsorbed layers of catalase and anticatalase which will be reviewed later. Some of the data, however, are of some concern for the enzymatic activity of successive layers. First, an anticatalase layer 50 to 60 Å thick, adsorbed on the catalase, did not diminish the activity of the enzyme. Second, the thickness of a second layer of catalase adsorbed on the anticatalase was only 10 Å, the order of an unfolded protein. Third, the activity of the system catalase—anticatalase—catalase was the same as that of catalase—anticatalase. One might conclude that the second layer of catalase 10 Å thick was inactive.

## 4. Saccharase

Experiments carried out by Sobotka and Bloch (16) with films of saccharase showed that films 45 Å thick retained their full enzymatic activity. The films were spread at the air-water interphase, transferred on slides, and subsequently tested with a drop of sucrose. Because of the solubility of the active films observed it is impossible to decide whether the enzyme may possess any appreciable hydrolytic activity

in the deposited state. Also in spite of the spreading technique used, the very thick layers obtained show that unfolding was far from complete. Sobotka's claim (5) that "the chemical nature of the enzyme is so limited that the very thick layers obtained cannot be taken to indicate unspread material" is hard to justify. If the enzyme is a protein, the thickness obtained is too large to indicate an unleafed molecule and consequently the results obtained do not furnish evidence for an enzymatic activity of an unfolded protein molecule.

#### V. IMMUNOLOGICAL REACTIONS

Porter and Pappenheimer (17) demonstrated that pneumococci polysaccharides adsorbed on slides react specifically with antibodies. Tests were carried out in the following way: Metal slides covered with an optical gauge of barium stearate, according to the method of Blodgett and Langmuir, were conditioned with dilute solutions of thorium nitrate and potassium silicate. Horse antibodies against Types I, II, or III pneumococci were adsorbed until maximum thickness increments were obtained (about 50 Å), then the slides were treated with a solution of homologous pneumococci polysaccharide. No appreciable increase in thickness was observed, which is easily understood if the long chain-like structure of the polysaccharide is considered. An increase in thickness of about 50 Å on subsequent treatment with homologous antibodies showed that adsorption of the polysaccharide had occurred. Three double layers of alternate antibodies and polysaccharides could easily be built up. In similar experiments with rabbit antisera the thickness of the adsorbed layer of antibodies was from 15 to 30 Å.

Investigations of the same kind were carried out by Harkins, Fourt and Fourt (15). Adsorbed layers of catalase 55 Å thick could adsorb a layer of anticatalase 50 Å thick. Alternate layers of catalase and anticatalase could be adsorbed, the thickness of the anticatalase layer being always 50 Å whereas all the catalase layers but the first one were 10 Å thick.

In these experiments dealing with adsorbed molecules, persistence of the immunological activity is not too surprising.

Work carried out with unfolded antigen or antibody molecules has a much greater significance for the understanding of the antigen-antibody reaction. Such experiments will tell whether the steric configuration of the antigen or the antibody is necessary for the immunological reaction or whether a two-dimensional pattern is a sufficient condition.

Danielli, Danielli and Marrack (18) were the first to investigate this question. They spread a film of antibodies against pneumococcus Type II and injected the corresponding polysaccharide in the fluid of the

trough at a concentration of 1/500,000. No difference could be detected in the area-pressure curve obtained before and after injection of the polysaccharide. Negative results were also obtained when they attempted to demonstrate by surface potential measurements a reaction between a film of spread globulin and the homologous rabbit antiserum dissolved in the substrate. Since then, a number of workers have demonstrated that films of unfolded protein antigens do react specifically with antibodies and also, in one case at least, that films of unfolded antibodies have not lost their property to combine with homologous antigens. Bateman, Calkins and Chambers (19) spread egg albumin and, after transfer on slides and treatment with different rabbit antisera, reported an increase of 69 Å with a homologous serum against 20 Å with a normal serum. No figure was given for the thickness of the egg albumin film. Films with the Lancefield M substance from hemolytic streptococci also reacted specifically with homologous sera. The thickness of the spread films (14 to 20 Å) indicates that complete unfolding had not occurred. An increase of 200 Å was observed after adsorption of undiluted homologous serum whereas the same serum diluted 1/300 gave an increase of 60 Å.

Rothen and Landsteiner (20), using the same technique, investigated the immunological specificity of spread films of egg albumin, heat denatured egg albumin, human albumin, horse albumin, native and denatured horse globulin. As many as thirteen different anti-egg albumin rabbit sera were tested. Clear-cut specificity was observed in all cases. The increase in thickness following adsorption of anti-egg antibodies on slides covered with one or two monolayers of egg albumin (8 to 10 Å per layer) varied from 40 to 55 Å. In the case of horse and human albumins and horse globulin the specific increase was between 30 and 40 Å. No difference was observed in the specific reactivity of films from native or from denatured egg albumin. However, anti-denatured egg albumin sera gave consistently higher thickness increments than anti-native egg albumin sera. Strong cross reactions were observed between films of horse and human albumins (8 Å thick) and the corresponding rabbit immune sera. But when the films were tested with sera diluted 1/10 in saline solution the cross reactions were abolished and at a dilution 1/200 a specific adsorption could still be demonstrated. In contrast to the findings with egg albumin, films from native and denatured globulin showed marked differences in their reactivity. A denatured globulin film reacted less with antisera to the native protein than to the denatured one, and *vice versa*.

On standing on the trough, films from native globulin were completely inactivated in about 4 hours. If, however, 2 minutes after spreading

they were compressed to 25 dynes, they retained their specificity for at least 16 hours. Films of egg albumin and horse albumin kept their immunological properties when left on the trough under no compression for 16 hours or more.

The same authors investigated spread monolayers of antibody molecules against pneumococci Types I and III. They demonstrated that a film of unfolded molecules 8 to 12 Å thick could react specifically with the homologous polysaccharide. The slides on which the unfolded films of antibodies had been transferred were covered with a drop of solution containing the polysaccharide, then washed, and subsequently treated with the antisera. The results are summarized in Table I.

TABLE I

*Reaction of Specific Polysaccharide with a Monolayer of Pneumococcus Antibodies I and III Spread at pH 7.3 (Veronal Buffer)*

Film anti- bodies 8 to 10 Å thick	Time between spreading and transfer  <i>min.</i>	Increase in thickness in Å after adsorption of polysaccharide		Increase in thickness in Å after adsorption of antibodies	
		I	III	I	III
I	60	2	—	45	—
I	60	—	2	—	15
III	120	3	—	8	—
III	120	—	2	—	46

This is the only case reported so far where an unfolded film of antibodies retained its immunological characteristic.

Experiments carried out with films of the globulin fraction of antisera for egg albumin and metakentrin (21), a luteinizing hormone isolated from swine pituitary glands, were negative. However, since the whole globulin fraction of the immune sera was used for spreading the film it may be argued that the film consisted mainly of inert globulin molecules and that no conclusions can, therefore, be drawn from negative results.

Chambers, Bateman and Calkins (22) used the following technique to demonstrate that a "nucleoprotein agglutinin" liberated from hemolytic streptococci by sonic disintegration, as well as a phosphorus-free protein derived from the same organism formed spread films which retained their immunological specificity. The antigens spread at different pHs (2, 4, 5, 7 and 8) were transferred on glass slides which were incubated for 2 hours at 56° in a broth suspension of the homologous organism in the presence of homologous or heterologous sera. After

rinsing, drying and staining the bacteria agglutinated on the slides were counted. The counts indicated that the transferred films were still immunologically active. Complete unfolding of the phosphorus-free protein occurred only at about pH 4.5. At pH 7.0 a film twice as thick was obtained.

Two series of experiments were carried out, in one of them the slides were covered with a double layer by immersion and emersion (AB layers) and in the other only one layer was deposited on the way down (A layer). The counts of agglutinated bacteria were much larger for the slides coated with an AB double layer than for the A layer. Some of the results are summarized in Table II. The figures in the third column represent the ratios of the number of counts for slides covered with a double layer AB and a single layer A.

TABLE II  
*Activity of Films of a Protein from Hemolytic Streptococci*

pH	Thickness per layer	Ratio of counts, AB/A
2	14.0	1.9
4.5	11.5	10.0
7	24.5	2.2

The authors concluded that these results demonstrated a dorsal-ventral asymmetry in the reactivity of the film, a B layer being more active than an A layer. These conclusions, however, are far from certain but are worth considering in detail. First of all, the comparison is not between an A and a B layer but between an AB and a B layer. It was tacitly assumed by the authors that the underlying A layer had no influence and consequently the difference in reactivity could only result from a different orientation of reacting groups in the A and B layers.

It is true that for many proteins, egg albumin or metakentrin for instance, the number of deposited monolayers has no influence on the thickness of subsequently adsorbed homologous antibodies. An increase in thickness of 30 Å follows adsorption of an antimetakentrin rabbit serum whether there is a single layer (8 Å) or a double layer (16 Å) of metakentrin on the slide (21). No difference can be noticed in the behavior of a single film of egg albumin, bovine albumin or metakentrin depending on the mode of deposition (downward or upward). However, as recently reported (23) the amount of adsorbed rabbit antibodies

against bovine albumin increases with the number of underlying monolayers of bovine albumin, as indicated in Table III.

TABLE III  
*Specific Reaction of Films of Bovine Albumin with Rabbit Antisera*

Number of bovine albumin monolayers	Increase in thickness in A after adsorption of	
	Antibovine albumin serum	Antiegg albumin serum
1	39	4
2	57	4
4	104	-3
6	136	-8
8	149	—

It is then perfectly possible that the greater reactivity of the AB layers observed by Chambers and co-workers may be accounted for by the presence of two layers instead of one and that the supposed orientation resulting from an upward or downward deposition does not take place. There is so far no satisfying evidence demonstrating a dorsiventral immunological activity from film experiments.

The question as to why, in the case of bovine albumin films, the amount of adsorbed antibodies increases with the number of underlying monolayers is of importance. First, it seems probable that the antibody molecules are piled up on top of each other in the thick layers of antibodies adsorbed on four double layers of bovine albumin. If this is true, the same process might take place in other cases. It has often been reported that undiluted immune sera gave much thicker layers of specifically adsorbed material than diluted sera. Bateman, Calkins and Chambers (19) found increments in thickness of 200 A and 60 A with undiluted and diluted serum, respectively. These variations have usually been assumed to result from a different orientation of the adsorbed antibody molecule which in the case of the rabbit antibody has approximately the dimensions of  $40 \times 270$  A. Great variations in the thickness of adsorbed layers of antibodies have also been observed by electron microscopy. For instance, Anderson and Stanley (24) reported an adsorbed thickness of 225 A of rabbit antibodies on the surface of tobacco mosaic virus molecules but they also have observed much smaller increments.

Mudd and Anderson (25) reported layers of antibodies on the surface of bacilli varying from 167 A on *Bacillus subtilis* to 24 A on *Eberthella*



*typhosa*. Mudd (26) came to the conclusion that "measurements of the thickness of the films deposited under various conditions are compatible with their interpretation as monomolecular films in which the ellipsoidal antibody-molecule is oriented with its long axis either approximately parallel or approximately perpendicular to the plane of the antigenic surface depending upon concentration and other experimental conditions; however, the alternative possibility that the observed change in thickness of deposited film with antibody concentration may be due to secondary adsorption of non-specific protein from the antiserum upon the primary antigen-antibody complex has not thus far been excluded." A third possibility could then be added, as we have seen, namely, that the observed change in thickness may be accounted for by more than one layer of antibody molecules similarly oriented. The findings of Freund (27) that collodion particles treated first with diphtheria or tetanus antitoxin and then with diphtheria or tetanus toxin were more toxic than particles treated with toxins alone might also be explained by assuming more than one layer of adsorbed material.

The question arises as to the nature of the forces which would immobilize the top layer or layers of antibody molecules. It may well be that the first adsorbed layer of antibody is so strongly polarized by its binding to the antigen layer that this induced polarization makes possible the specific adsorption of a second or even more layers. Analogous instances are known where multilayers can be built up in such a way. At a pH near the isoelectric point, multilayers of insulin up to 450 Å can be adsorbed on a layer of protamine 30 to 50 Å thick, the thickness of the protamine layer being of minor importance (28). Also, uranyl ions adsorbed on slides covered with barium stearate make possible the transfer of a large number of successive layers of protein from the water interphase which otherwise would not have adhered to the slide. This demonstrates that the uranyl ions strongly polarize the first layer, which in turn polarizes the second and so on along the successive layers (23).

Other experiments recently described (23) point to the presence of possible long range forces between films of antigen and antibody molecules. When films of barium stearate or of heterologous proteins are deposited on top of antigen films they do not prevent a subsequent specific adsorption of homologous antibodies. The obvious explanation that the screens of stearate or protein have holes through which the antibody molecules can reach the antigenic film is not satisfactory in view of the closely packed structure of such screens in relation to the size of the antibody molecule (molecular weight  $\sim 160,000$ ). Furthermore the rate of the reaction is of the same order of magnitude whether there is

a screen or not. The nature of the screen does not seem to play an important role since this phenomenon of specific fixation at a distance occurs as well with screens of the same order in thickness of plastic film "Formvar" which do not show any discontinuity in the electron microscope ( $\sim 30$  A) (29). It may be assumed that the effective range of action between a film of antigen and antibody molecule extends to an order of hundreds of A instead of a few A, as in the case of forces between individual atoms.

Experiments recently published (29) indicate that long range interaction may take place in the mechanism of enzymatic activity. For instance, crystalline trypsin, as well as pepsin, is capable of exerting its proteolytic action on antigenic films deposited on metal slides in spite of intervening screens of barium stearate or "Formvar". In the case of bovine albumin films, the greater the number of underlying monolayers, the greater the thickness of the barrier necessary for protection against enzymatic action. A screen of Formvar 65 A thick prevented inactivation in 10 minutes of one double layer of bovine albumin whereas the screen's thickness had to be 150 A when there were three double layers of bovine albumin. Analogous experiments were performed with adsorbed layers of the polysaccharide from pneumococcus III and the specific enzyme capable of hydrolyzing the said polysaccharide. Hence, this apparent long range action is not limited to proteolytic enzymes.

These experiments open a new vista on the mechanism of the enzymatic action. Enzyme molecules need not be in contact with the molecules subjected to disintegration. This long range interaction might take place through resonating extended oscillators, the presence of which is likely to occur in large molecules as suggested by London (30).

## VI. PHYSIOLOGICAL ACTIVITY OF FILMS

The testing of the physiological activity of unfolded proteins is a difficult problem since it usually must be made in animals and consequently the extended two-dimensional pattern of an unfolded protein is *ipso facto* changed. However, if a collected spread film of a protein is found inactive, it is logical to conclude that inactivation occurred at the time of unfolding. Three physiologically active proteins have been investigated so far (21). They are (1) metakentrin, a gonadotropic hormone of the anterior lobe of the pituitary gland, (2) the oxytocic pressor hormone of the posterior lobe, (3) crystalline insulin. These substances were particularly suitable on account of their purity and of the extreme sensitivity of the biological test, the limit of the test being 1  $\gamma$  protein nitrogen for metakentrin (anterior prostate of immature hypophysectomized rat) and 0.06  $\gamma$  for insulin (convulsions

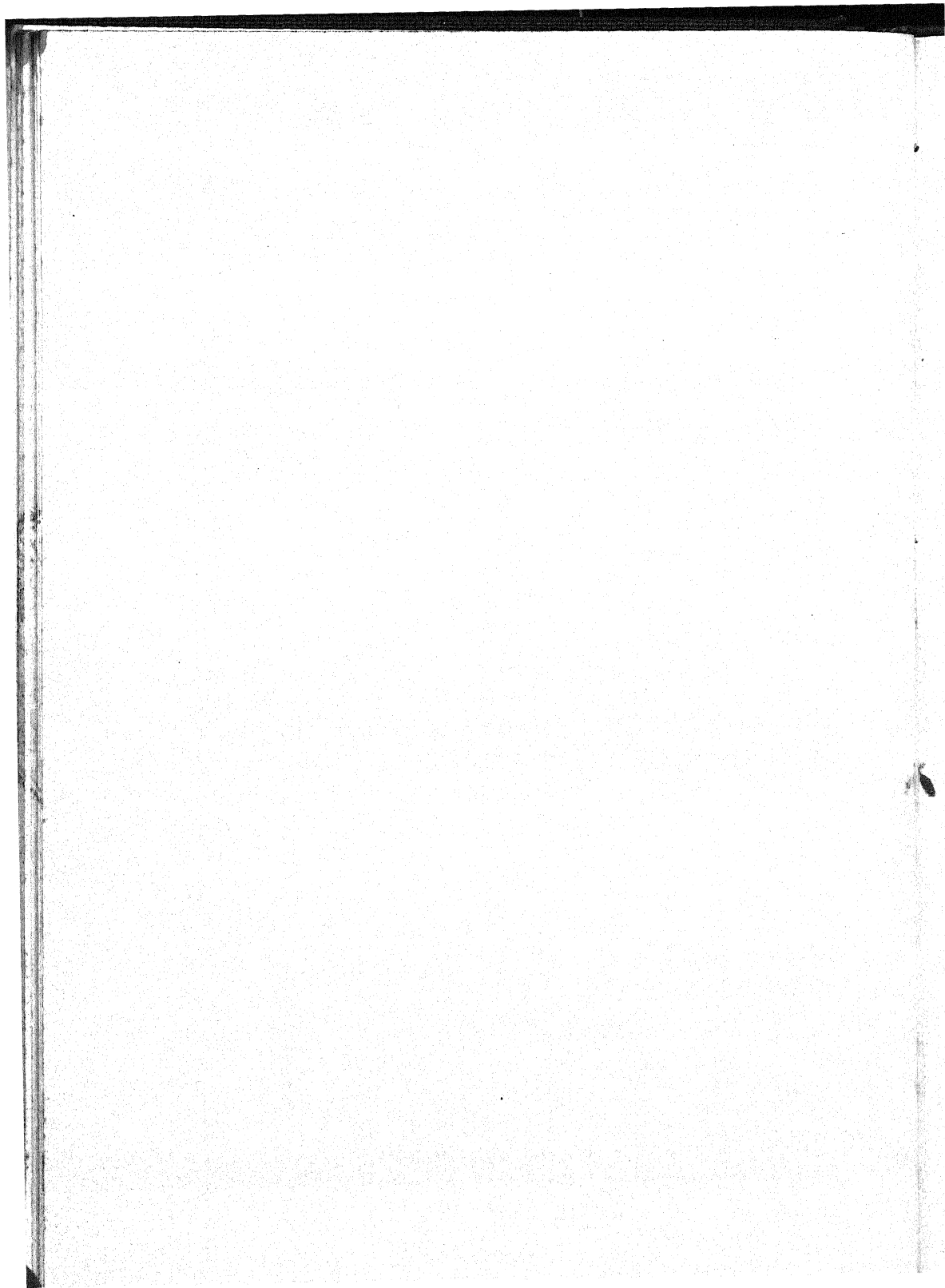
in the starved albino mouse). For the posterior lobe hormone the threshold is dependent upon the test 0.05  $\gamma$  N (oxytocic effect assayed by action on the fowl's blood pressure or on the isolated guinea pig uterus), or 0.01  $\gamma$  N (pressor effect estimated by diuresis-inhibition in the rat).

These three proteins spread on buffered solutions at their respective isoelectric point (7.4 for metakentrin, 4.6 for posterior lobe hormone, and 5.6 for insulin) form expanded films 7 to 9 Å thick. Such films were collected at the water interphase by means of a platinum loop enclosing an area of 8 cm.<sup>2</sup> The amount of material estimated by the area collected agreed with the value given by nitrogen determination. Assays of collected films of metakentrin demonstrated that most (> 90 per cent) if not all of the physiological activity had disappeared. The same was true for the activity of the films of the posterior lobe hormone, more than 98% of the oxytocic and pressor activity was destroyed by unfolding the original native protein. The results were strikingly different for insulin where no loss of activity was observed following unfolding. It could be argued that a film of spread insulin molecules is inactive but that the molecules after the collapse of the film would after dispersion in water regain their original configuration and activity. Collected films at pH 5.6 gave an opalescent solution showing that strong aggregation took place. Such suspensions became clear on acidifying the medium. Full activity was found for the suspension of insulin molecules as well as for the acid solution. These facts point against a reversible activation-inactivation process. Thus it seems logical to conclude that the activity of insulin is localized in a relatively small two-dimensional pattern.

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# The Chemical Determination of Proteins

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## I. INTRODUCTION

The analyst who desires a method for the determination of the protein content of plant or animal material is confronted with a voluminous and confusing literature. No better indication could be found of the unsatisfactory state of analytical development in this field. The reasons are apparent. Proteins form a very diverse group of similar compounds of extraordinary complexity, with widely different compositions and properties, yet difficult to separate completely, to purify and to dry. Their amphoteric nature, high adsorptive capacity, hydration properties and sensitivity to electrolytes cause them to vary widely in behavior depending on the composition, pH and temperature of the solvent medium (1). Moreover, they usually occur in mixture with each other in variable concentration ratios and in various solid and dissolved states. The analyst may be concerned with the content of a particular protein in such a mixture, or commonly, he wishes to know the total protein content when that content is itself made up of a mixture of variable and uncertain composition.

The analytical problem has several aspects, which may be resolved as follows:

1. Relatively uniform material such as blood, cerebrospinal fluid, or urine requires rapid analysis for purposes which usually do not require high accuracy, but should have a considerable precision or reproducibility.

2. Complex samples such as foods, or animal and plant tissues must be analyzed for their total protein content or its fractions. Some research purposes may require as great accuracy as possible in which case the problem is difficult. If only routine approximate analyses are desired the difficulties are minor.

3. Specific proteins, or individual protein constituents of a mixture may require determination. In these instances, special methods are often available, in which advantage is taken of some specific property of the protein, *e.g.*, the color of hemoglobin and its derivatives, enzymatic activity of a protein enzyme, an immune reaction, or blood coagulation by thrombin.

This review deals with the problems of 1 and 2 because these are the categories of widest general analytical interest. The problems of fractionation and precipitation of proteins, on which there is much diversity of practice and results will also be considered briefly. In general the proteins which exhibit some highly selective property present a simpler problem, though here also there is a diversity of methods and opinions.

At the present time, all methods for protein analysis are empirical by nature, or by virtue of technical necessity. Isolation and direct weighing of the protein would provide an absolute method were it possible to show that the weight determined represents pure, unaltered, completely dry protein. All methods of separating and drying proteins have been more or less empirical, so that uncertainties of a few per cent are usually unavoidable. Other methods depending on the determination of some constituent or some property of the protein which parallels its concentration are inherently empirical, and must be arbitrarily calibrated.

The precision of protein analysis may be made as great as the technical procedures and degree of standardization permit, but the absolute accuracy is always subject to uncertainty.

The important general methods of approach to the analysis of proteins will be considered separately in the following sections.

## II. ANALYSIS FOR CONSTITUENTS

Since no specific method is available for determination of protein as such (with the exception of special proteins having unique properties, such as some enzymes, etc.), the most common procedures depend on

determining an element or group contained in the protein molecule. In order to use this type of method, the analyst must either (a) know the composition of the protein with respect to this constituent, or (b) standardize the method against some other procedure about which more information is available.

Methods based on analysis for constituents of the protein molecule include those for determination of the elements carbon and nitrogen; certain amino acids such as tyrosine; the biuret grouping; and formol binding groups. Individual proteins may sometimes be determined by virtue of special constituents such as the iron in hemoglobin (2, 3) or the iodine in thyroglobulin. All of these methods require that the constituent determined be present in the sample entirely in the protein fraction. Thus, protein must be separated from all other organic matter and carbonate if it is to be determined from its carbon content, and from all other nitrogenous constituents if the Kjeldahl method is the basis of analysis. The common practice of analyzing feeds and vegetable material for protein by determination of the total nitrogen is always subject to considerable uncertainty on this account. The presence of alkaloids, amino acids or other nitrogen containing materials is not unlikely in such material, though as a rule, the quantities are small as compared to the protein content. Because of the variability of properties of the various proteins, no generally applicable method of extracting them from complex mixtures is available. In well studied systems specialized extraction procedures may be applicable.

### 1. *Elementary Constituents*

*Carbon* analysis was used for determination of protein by Hoagland and Fischer (4) who adapted the Van Slyke manometric method for this purpose. They analyzed the protein in as little as 0.1 ml. of serum. Advantages claimed for the method included, (a) a relatively constant factor for carbon content of different proteins; (b) a high percentage of carbon in protein, a factor which minimized experimental errors; and (c) an easier digestion than that for nitrogen determination. Separation of the protein from all other carbon containing constituents of blood serum was obviously necessary. For this, the standard tungstic acid precipitation procedure was used, both for total protein determination and for the fractions separated with sodium sulfite by the method of Campbell and Hanna (5). There is little available information on which to base the choice of a precipitant which will separate protein from all other organic materials. Polonovski, Warembourg and Cuvelier (6) employed a somewhat similar procedure based on an earlier method of Boulanger and Warembourg, in which serum was pipetted directly into chromic



acid and oxidized. A similar sample from which the globulin had been precipitated with magnesium sulfate was analyzed in parallel. The assumption that the difference in the two values represented the globulin may have been approximately correct. The necessary correction of the figures for carbon other than that of the protein is certainly not easy to evaluate exactly. Boutroux (7) called attention to the failure of the above authors to separate lipoids in determining the albumin-globulin ratio. A similar but somewhat better procedure was described by Cordebard (8) who performed a preliminary protein separation by means of metaphosphoric acid. The excess of chromic acid used for the oxidation was determined iodometrically, a procedure which is subject to considerable errors (9).

The fact that protein has seldom been analyzed by determination of the carbon contained in it does not indicate that this is an undesirable procedure. On the contrary, it has several important advantages over other methods for precise analysis, when the sample is such that complete separation of protein from all other sources of carbon may be accomplished. With the exception of a very few techniques such as that of Van Slyke, Page and Kirk (10), accurate carbon analysis is itself not simple which probably explains in part why it is not used more widely in this connection.

*Nitrogen* analysis for protein determination has been so commonly used that a complete discussion of the literature is entirely beyond the scope of this review. It is usually assumed that a mixture of pure proteins will contain 16% nitrogen. Thus the protein content of a sample is obtained by multiplying the determined nitrogen by the factor 6.25. For approximate analysis of foodstuffs and other very heterogeneous mixtures, containing an unknown distribution of proteins of unknown composition, it is not easy to suggest a procedure which is more practical. Even for pure proteins, and for simple mixtures of proteins, the nitrogen, determined by some modification of the Kjeldahl method is almost universally used as the standard by which all other methods are evaluated. Despite its wide acceptance and use, the method is fraught with difficulties of a serious nature. Basically, they consist of (a) the inherent difficulty of separating total protein or protein fractions completely from all other nitrogenous materials; (b) the uncertainty of the nitrogen content of the particular protein fraction studied; and (c) difficulties in the Kjeldahl determination of nitrogen.

Separation problems, (a), are common to many types of protein analysis and related to the problems of fractionation. These will be discussed in a later section. The uncertainty of nitrogen content, (b) arises first, from the fact that certain proteins are rich in glycine or lysine,

arginine and histidine, while others are almost devoid of these high nitrogen content amino acids, and second, from the uncertainty as to what constitutes a perfectly dry, unaltered protein (11). In 1931, tables issued by the U. S. Department of Agriculture (12) showed that many of the conversion factors for various foods and feeds varied considerably from the single factor commonly used. Casein, which approximates average composition and which has been extensively investigated, has been reported by Block (13) to contain 14.7% nitrogen (uncorrected for moisture and ash); 14.6% (uncorrected) or 15.5% (corrected for calcium and phosphorus) by Ramsdell and Whittier (14); 15.7% (corrected) by Chibnall, Rees and Williams (11); and 15.9% (corrected) by Jonnard (15). Adair and Robinson (16) found 15.60% nitrogen in carefully dialyzed and crystallized serum albumin. They report a compilation of previous values ranging from 15.1% to 16.04%. It is a fact that the analyst rarely knows with certainty the correct conversion factor to use for any given protein which he is analyzing. Since the nitrogen content of individual proteins may vary from about 14 to 19%, it is evident that analysis of protein mixtures of unknown composition may be determined only approximately by use of any empirical factor which has not been carefully determined for the particular system.

Difficulties in the Kjeldahl determination of nitrogen (c), center chiefly on the problem of digestion. Variable factors known to influence the completeness (and speed) of conversion of protein nitrogen into ammonia by sulfuric acid digestion include the following sources of error:

1. The chemical form of the nitrogen in the sample. All proteins contain their nitrogen in several known types of linkage. Of these, at least the nitrogen of tryptophan and lysine (17), and histidine (11) is known to be difficult to convert to ammonia. This source of error may be enhanced if some of the nitrogen is converted during the digestion into a resistant form or one which is volatile under digestion conditions.

2. Presence in the digest of salts which raise the boiling point. The sodium or potassium sulfate which is customarily added to raise the boiling point of the digestion mixture increases the speed and completeness of the digestion but an excessive ratio of the salt to the acid used may lead to the loss of ammonia through heat decomposition of  $\text{NH}_4\text{HSO}_4$  (18, 19). Many analysts have employed phosphates instead of part or all of the sulfate (20, 21, 22, 23). When up to ten-sixteenths of the total salt was  $\text{K}_2\text{HPO}_4$ , the digestion was considerably shortened and apparently was more complete. The use of more phosphate than this led to low results (24).

3. Use of oxidizing agents. The addition of potassium permanganate at the end of the digestion to complete the oxidation is an old procedure

and one which apparently yielded satisfactory results in the hands of Osborne (25) and Sørensen (26). More recently, potassium persulfate (27, 28), perchlorates (29, 30), and hydrogen peroxide (31, 32) have been used for this purpose. Their influence on the digestion time, completeness of recovery and loss of nitrogen have been the subject of considerable controversy in the analytical literature.

4. The use of catalysts. Mercury, copper and selenium have been widely employed as catalysts for the oxidation of organic material in hot sulfuric acid. So many contradictory findings have been reported, that the literature on the subject is both voluminous and confusing. Nearly all of the likely elements of the periodic table, 39 in one single study (33), have been tested for their effects on the Kjeldahl digestion (34). Of the 10 or 12 (33) which were found to catalyze the reaction, only the three mentioned, and iron (21, 23, 35) have received much serious attention in recent years. Several elements exert a retarding effect on the digestion, or cause loss of nitrogen (33).

5. Length of digestion time. Too often, analysts have assumed that clearing of a Kjeldahl digest signified virtual completion of the digestion. While the fact that there is little relationship between the time of clearing and the total necessary digestion time (36) has long been a matter of record, it is only lately that wide-spread attention has been given to this factor (37, 38, 39, 24, 11, 15).

6. Interfering materials. The interference of ammonium salts often used for fractionation of proteins is so obvious as to call for no comment here. Less apparent is the possible interference of elements or compounds present as impurities in the sample, which may cause loss of nitrogen or incomplete digestion, and particularly certain compounds used in the precipitation of the protein such as sodium tungstate or phosphotungstic acid which were found by Jonnard (15) to cause low and variable recoveries of nitrogen.

7. Use of reducing or hydrolyzing agents prior to digestion. Reducing agents have been regularly employed to convert oxidized forms of nitrogen into a reduced form which is determinable by the Kjeldahl method. One of the oldest and still widely used methods involves the use of phenol (40) or salicylic acid (41) followed by one of a number of reducing agents, the most common of which is sodium thiosulfate (42). Though proteins do not normally contain oxidized forms of nitrogen, some of the more difficultly converted nitrogen of the protein is more completely recovered if it is exposed to the action of reducing and hydrolyzing agents such as hydriodic acid (43, 44, 15), before being digested.

In considering the above sources of error in the Kjeldahl digestion it

is apparent at once that nearly all investigators have studied a portion of the sources of variation without considering all of them simultaneously. Consequently the results of various studies are not strictly additive and may give contradictory results because certain factors were not controlled. Without considering other forms of nitrogen than those occurring in the protein molecule, the most important factors appear to be the catalyst, the time of heating and the use of additional reducing or oxidizing agents. Secondary factors include the hygroscopicity of protein samples which prevents accurate weighing of thoroughly dried samples, an effect which was recognized very early by Osborne and others and was recently carefully studied by Chibnall, Rees and Williams (11).

As *catalyst*, both copper and mercury compounds have long been used and the consensus of opinion certainly indicates the superiority of mercury over copper, though an additional step is necessary in its use, *viz.*, its precipitation to decompose the ammonia complex which it forms. Following the introduction of selenium and its compounds as catalysts by Lauro (45), many conflicting results of its use were reported. Disagreements of different investigators is evidently due, at least in considerable part (a) to the confusion of clearing time with digestion time, and (b) to a frequent failure to keep the quantity of selenium lower than was customary with other catalysts. Bradstreet (46) has specified the upper limit of 0.25 g. Se for digestion of a macrosample, with or without iron or copper catalyst. Above this limit recoveries were reduced. Osborn and Wilkie (33) found that selenium was effective only in ordinary amounts or under less violent conditions than were frequently used. Osborn and Krasnitz (47) reported that selenium caused loss of nitrogen unless the acid content was kept high. Dalrymple and King (37) advise against prolonged digestion with selenate as a catalyst because the recovery rises with time to a maximum and subsequently diminishes, confirming Sandstedt (48) who found loss on prolonged digestion. However, the consensus of opinion certainly indicates that selenium in proper quantities combined with sufficient acid and an amount of sodium or potassium sulfate which is not excessive, is an effective catalyst (49, 50, 51, 52, 53, 47, 46, 38, 34). The most pronounced effect of selenium is a rapid clearing of the digest (54, 55). Many investigators have realized that clearing was no criterion of complete digestion (55, 24, 38) and the frequently used method of digesting for 1.5 times the clearing time (56) has recently been called clearly into question (55, 57, 19). This was most strongly emphasized by Chibnall, Rees and Williams (11) and by Jonnard (15), who recommended from 8 to 16 hours digestion after clearing. It was necessary to take special precautions to maintain the acid content high throughout the digestion, since long digestion time

has been found unsuitable by several investigators who may have lost nitrogen due to use of insufficient acid (47, 24). The available evidence certainly indicates the superiority of selenium over other catalysts as a clearing agent and it probably is superior also in reducing over-all digestion time. Mercury, on the other hand is certainly as dependable or better for obtaining maximum recovery of nitrogen (33, 54, 47, 58, 59). The combination of mercury with selenium, properly used, is almost certainly superior to either alone. Particularly significant results on the mechanism of selenium catalysis have been published by Sreenivasan and Sadisivan (60) and valuable general studies on the digestion process were made by Milbauer (34) and Kahane and Carrero (61).

Combinations of catalysts have been widely and effectively employed. Osborn and Krasnitz (47) have found the combination of mercury and selenium to be the most effective with refractory material, a result which has been confirmed by others (62, 63, 60, 55, 53). Copper, mercury and selenium in combination have been found very effective by several investigators (64, 65, 62, 56). Copper and selenium in combination are more effective than copper alone (38, 50, 66). The mercury-selenium combination in the form of mercuric selenite has been recommended (67). The use of copper selenate (37) and of copper selenite (68, 69, 22) as a convenient combination of copper with selenium has been successfully adopted by various investigators. It is very unlikely that the various catalysts are comparable in efficiency when compared with each other in analyzing different forms of nitrogen. Much of the divergence of opinions on the subject arise between investigators working with different types of material. Relatively few laboratories have produced comparative data on analysis of the same types of sample by the Kjeldahl and Dumas methods. Though it is known that the Dumas analysis is not strictly dependable with certain compounds such as cytosine and pyrimidines (70, 71), it appears that the Kjeldahl procedure has often given lower results than the Dumas. Anne (72) reports that the Kjeldahl method gave lower results than the Dumas on all products tested except humic acids. The differences ranged from 0.35% in the case of gluten up to 9.9% for wheat flour. Lemoigne, Desveaux and Monguillon (19) also found lower results with the Kjeldahl than with the Dumas analysis of agricultural products with most, but not all samples. Wheat flour again yielded the greatest discrepancy. Dumazert and Marcelet (67) compared the two methods on urine, proteins, tissue powders and feces. The Kjeldahl method always gave lower results unless mercuric selenite was added in which case the differences were reduced or became negligible. More complete studies in which all the known variable factors are considered simultaneously should definitely be made, and such

studies are particularly needed for microdigestions on which there are few or no careful studies of these factors.

The use of certain *oxidizing agents* such as potassium persulfate, perchloric acid, and potassium permanganate has led to much difficulty. Though Osborne (25) and Sørensen (26) both used potassium permanganate to complete their digestions, Osborn and Wilkie (33) and others (24) have found that it interferes with the accuracy of the determination. Potassium persulfate has been recommended and used frequently, particularly in the clinical analysis of blood and similar materials (28, 73). Few extensive and critical studies are available on the use of this material. Robinson and Shellenberger (27), in comparing the micro-Kjeldahl method with the official Kjeldahl-Gunning-Arnold method on flour, wheat and corn, used potassium persulfate without noting difficulties, while Stubblefield and De Turk (23) report consistent loss from this reagent in the analysis of alfalfa samples. Though Pepkovitz and coworkers (30) and others report satisfactory use of perchloric acid, Kaye and Weiner (74) using the same procedure obtained consistently low results. Wicks and Firminger (75) tested the use of this reagent and also observed consistent loss of nitrogen, which was confirmed by Jonnard (15). This loss has been attributed to formation of free nitrogen (76) and possibly to undecomposed amines (75). Hydrogen peroxide is the only oxidizing agent which has not been reported to cause loss of nitrogen (31, 64, 77). That this may be due in part to the small number of careful studies of the reagent would appear to be a definite possibility. The author has employed hydrogen peroxide consistently in the analysis of pure compounds without noting any consistent loss of nitrogen. However, this material contains nitrogen, probably in the form of acetanilide, added as a preservative, and its use must be rigidly controlled and blanks analyzed with the same amount of hydrogen peroxide as used in the samples if high results are to be avoided (78). If the reagent causes loss of nitrogen from some samples, it might well be compensated by using rather large amounts of peroxide and not considering the amount of nitrogen unintentionally added. In general, it is evident that the greatest care is necessary in the use of any oxidizing agent, and the standard procedure employed should be carefully checked.

The use of *reducing agents* prior to digestion is ordinarily only necessary when oxidized forms of nitrogen are present. Hydriodic acid and red phosphorus, which was introduced in this connection by Friedrich (43), serves both as a reducing and hydrolyzing agent which makes possible by appropriate method of use, the determination of all forms of nitrogen except nitrate and nitrite. According to Jonnard (15) some of the nitrogen of proteins is not completely recovered unless the modi-

fication of Clark (44) or the simpler use of hydriodic acid alone (79) combined with selenium catalyst and longer digestion time is employed. Further investigation of the necessity of using this relatively slow and difficult method should be carried out, in view of the large number of studies and widespread use of simpler methods.

The determination of ammonia after the regular or modified Kjeldahl digestion presents rather less serious problems than those already discussed. The advantages of the micro-Kjeldahl distillation (69, 80, 81, 82, 83) as compared with the macro-method, or even the semimicro-method are now generally recognized. A comparative study of the macro- and microscale determination in the analysis of flour, wheat and corn for their protein content was made by Robinson and Shellenberger (27). The micro-Kjeldahl method has been used for systematic plasma protein analysis (84, 85), saliva proteins (86), milk proteins (87), and cerebrospinal fluid protein (88).

Direct Nesslerization of the Kjeldahl digest has been perhaps more commonly employed (89, 90, 91, 92, 93) than has the regular distillation method. The color formed has been measured in a wide variety of colorimeters and photometers. Some time is saved by this procedure, and it has also been preferred for clinical purposes because of the habitual use of the colorimeter in clinical analysis. The procedure is certainly less accurate and less dependable than distillation, but it is also possible that the errors introduced are not larger than those already inherent in other portions of the analytical procedures. The formation of amines (61, 76) during digestion would undoubtedly introduce errors in this type of procedure. Schere (94) studied the distillation and Nesslerization methods comparatively and concluded that the latter checked the former closely enough for clinical purposes. An interesting variation which avoids many of the criticisms of the Nessler procedure is the use of hypobromite to oxidize the ammonia in the digest (95, 96). The excess hypobromite is determined iodometrically. The use of phosphomolybdic acid (96) in the digestion is probably undesirable (15).

## 2. *Amino Acids*

Amino acids having specific groupings which may be readily determined have offered a simple approach to protein analysis. Of these tyrosine (and tryptophan) have received the most attention. Little effort has been made to analyze for protein content by determination of the sulfur containing amino acids, cystine and cysteine. Amino acids containing a benzene ring react with hot concentrated nitric acid (xanthoproteic reaction) to form yellow nitro compounds which become orange on neutralization. Millon's reagent and that of Folin, particularly the



Folin-Ciocalteu reagent (97) form colored compounds with phenols (tyrosine) whose concentration may be measured colorimetrically.

The use of the phenol reagent for analysis of protein content was introduced by Wu (98). Greenberg (99) modified the procedure by introduction of the Folin-Ciocalteu reagent. Other modifications have been described by Greenberg and Miralubova (100), Johnson and Gibson (101), Minot and Keller (102), and Pressman (103). This is unquestionably one of the more empirical of the protein analytical procedures which have been developed. The content of reactive groups may only be established with pure proteins or known protein mixtures, and it will vary greatly between different proteins. This fact restricts the use of the method to well studied systems such as blood or cerebrospinal fluid. Moreover, the color intensity is greatly influenced by technical variations and analytical conditions (102, 103). Wokes and Still (104) found variations depending on the age of the serum analyzed. Herriott (105) found that a trace of copper sulfate altered the color intensity for protein and for non-phenol containing compounds, but not for amino acids. Tuchman and Sobotka (106) were unable to obtain agreement between the Wu method and the Kjeldahl analysis when the protein content was below 6%, as in the blood of edematous patients. Pressman (103) particularly, recognized the empirical nature of the method and made a careful study of the several variables which could cause analytical variations, so that the proper measures for standardization could be introduced. He found the method useful because of its sensitivity and speed when used with a simple and reproducible system, *i.e.*, specifically precipitated antibody globulin, to which the likewise empirical but faster physical methods were not easily applicable.

The determination of protein by nitration of its benzene ring containing amino acids (xanthoproteic reaction) has the single advantage of a simpler reagent than methods based on the Folin phenol reagent. The reaction is less sensitive and is somewhat difficult to standardize. Techniques for protein analysis by this means have been described by various authors (107, 108, 109).

### 3. *Biuret Grouping*

The biuret reaction, though long known and used as a qualitative test for proteins, has received wide attention as a quantitative method only in rather recent times. Riegler (110) was probably the first to use it in this way. The reaction would appear to be less subject to criticism as the basis for a colorimetric method for protein analysis, than nearly any other such method. It is given by all proteins in approximately the same intensity, and by few of the probable contaminants of



a protein solution (111), which is not true of most colorimetric or physical methods. In common with most other colorimetric methods, it is not affected by presence of ammonium salts used in fractionation. It has also been studied more thoroughly than about any other similar colorimetric method. The color must be standardized against known protein, or against another method (*e.g.*, the Kjeldahl analysis for nitrogen), and it is therefore not an absolute method. That its obvious advantages have been largely confirmed in practice is indicated by the large number of its applications in recent years. It is odd that the method has been used almost exclusively for analysis of simple systems such as blood and cerebrospinal fluid rather than for more heterogeneous systems such as food, feeds and tissues of plants and animals.

The most widely used procedure for determining protein by the biuret reaction seems to be that of Kingsley (112) though several earlier publications (113, 114, 115, 116) have appeared. Kingsley's applications were entirely to blood proteins. Campbell and Hanna (117), Sunderman and Schmidt (118) and Andersch (119) studied the application to cerebrospinal fluid, while Johnson (120) applied it to analysis of allergenic extracts.

More satisfactory studies of the method than those mentioned above were made by Robinson and Hogden (121) with the spectrophotometer, and by Pereira (122) with the Zeiss-Pulfrich step-photometer. Robinson and Hogden found that the intensity of the absorption at  $560\text{ m}\mu$  did not alter for at least 48 hours, but the density of the region at  $700\text{ m}\mu$  diminished. They also found that rabbit, dog and human sera gave the same transmission minimum at  $560\text{ m}\mu$ . It is interesting that one of the advantages claimed by Kingsley for his method, *viz.*, that he could get better results without preliminary precipitation of protein, was not confirmed by the above authors who specified a trichloroacetic acid precipitation as a necessary step in the analysis. These authors, as well as Pereira (122) state that it is necessary to use about 20% copper sulfate to prevent carrying down of protein. The latter author also added ox, horse and sheep to the list whose serum gives equal optical values for equal protein concentration. He found obedience to the Lambert-Beer law, and equivalence of trichloroacetic acid and tungstic acid as precipitants. Campbell and Hanna (117) introduced the very significant use of lyophilized protein as a standard. Mehl (123) who also studied the reaction spectrophotometrically, recommended the use of ethylene glycol to prevent precipitation of copper hydroxide. He reported that measurement in the ultraviolet at  $320\text{ m}\mu$  gave greater sensitivity than did the use of any line in the visible range. Plekhanov (124) described a volu-

metric modification of the biuret method, which, in its present state does not seem to offer any particular advantages.

#### 4. *Formol-binding Groups*

The increase in acidic groups which can be titrated with base, when amino acids, proteins and polypeptides are reacted with neutral formaldehyde, is the basis for the well-known formol titration methods which have proven quite serviceable in the analysis of  $\alpha$ -amino nitrogen of amino acids. It might be expected that proteins, which also have amino and carboxyl groups (as zwitterionen) might well be analyzed in an analogous manner. This expectation has not been realized satisfactorily, either because of the fact that in addition to a few free terminal  $\alpha$ -amino groups, the free amino groups of the protein are terminal groups of lysine, whose behavior is different from that of  $\alpha$ -amino groups, or because of erratic behavior of the protein molecule due to its complexity. Numerous old, and several more recent publications (125, 126, 127) have appeared in which the formol titration for protein analysis is described and even recommended as "rapid and accurate" (127). McDowell and McDowell (128), on reinvestigating their earlier procedure for casein analysis in milk (125) found their original conversion factor to be incorrect by about 8% and to vary by  $\pm 3\%$  under ordinary analytical conditions. They claim  $\pm 5\%$  over-all accuracy under highly standardized conditions, by a method which at best is not rapid. Considering that their system was one which was particularly favorable for this type of analysis, it is not surprising that the method has gained little popularity. Popova (129) using the standard formol titration claims an accuracy of 1%, but states that for accurate work, the Kjeldahl method must be used. Few analysts claim better than 1% accuracy for the Kjeldahl method, and the variation in listed conversion factors for single proteins is usually considerably greater than this amount when based on the nitrogen content. Another interesting procedure is that of Biguria and Foster (130) who formed formol-gels in a series of diluted samples, and determined the dilution at which air bubbles remained stationary in the gel after a 24-hour period of standing. The formol titration applied to protein analysis appears to offer some of the best illustrations of the analytical confusion often to be found in the biochemical literature.

### III. PHYSICAL METHODS

Proteins exert relatively large effects on certain physical properties of their solutions as compared with simple solutes dissolved in the same solvents. Among the physical properties which undergo variation depending on the protein concentration may be listed (a) specific gravity

(or density), (b) refractive index, (c) viscosity, (d) surface tension (and the related spreading on surfaces), (e) absorption of radiation, (f) electrical conduction properties, and (g) rotation of polarized light. Several of these properties have been made the basis for methods of analysis of protein solutions. The underlying assumption in all of them is either (1) that the effect of non-protein solutes is negligible in comparison with the effect of the protein on the property in question, or (2) that no appreciable uncorrected variation occurs in the non-protein constituents. To the extent that these assumptions are valid, the methods based on them are useful. Since some physical properties lend themselves to easy, rapid and frequently very accurate measurement, some of the most convenient and useful analytical procedures for relatively reproducible samples are based on them. It is important to note that the physical property itself can generally be measured quite accurately and reproducibly, but the relation of that property to the protein concentration may be uncertain, due to the failure of one of the basic assumptions.

### 1. *Specific Gravity*

Interest in determination of protein content of blood serum or plasma by means of specific gravity measurements, developed largely as a result of the publication by Barbour and Hamilton (131) of a simple micro-method for specific gravity determination with solutions. The time of fall or a 10  $\lambda$  drop of the sample through 30 cm. of a mixture of xylene and bromobenzene of a density slightly less than that of the sample, was measured. By comparison with a standard solution of potassium sulfate of known specific gravity, the unknown specific gravity could be readily calculated. With proper temperature correction, the limiting accuracy was  $\pm 0.0001$ . Moore and Van Slyke (132), depending chiefly on pycnometric determination of specific gravity, studied the application to blood serum and derived conversion factors for obtaining the protein concentration from the measured specific gravity. They found that a concentration of 7 g. protein per 100 ml. gave a density of about 1.027 which changed linearly by 0.0029 per gram of protein. The limiting accuracy of the falling drop method corresponds then to a limiting detectable concentration of about  $\pm 0.05$  g. per 100 ml. Moore and Van Slyke report a variation of as much as 0.6 g. protein per 100 ml. between the specific gravity method and the Van Slyke gasometric micro-Kjeldahl (133). This difference represented the sum of experimental variation in both procedures added to the uncertainty of the fundamental assumptions stated above, and the uncertainty in interpretation of the Kjeldahl method. Variations between the pycnometric and falling drop determina-

tions of specific gravity were in general less than 0.0003, showing that it was not the defect in determining the specific gravity by the falling drop method which led to the variation noted.

The method of Barbour and Hamilton has been applied with slight modifications and extensions by various investigators to the several phases of serum or plasma protein analysis (134, 135, 136). Modifications of the reference medium have been introduced by Kagan (137) who also extended the application of the method to whole blood (138); De Vries (139); Atlas, Cardon and Bunata (140); Phillips *et al.* (141) and others. The falling drop method in some of its various forms has not given uniformly good results in all hands and under all conditions, which is not unexpected in view of its inherent limitations and the many variations of samples and of individual techniques. The method or some of its modifications has been criticized particularly by Zozaya (142), Looney (143) and Quigley and Muraschi (144). Since Stokes' law applies to the falling drop method, and since viscosity is as important in the Stokes' law relationship as density, any factor which alters the viscosity of the medium would give rise to serious difficulty. This has been emphasized by Atlas, Cardon and Bunata (140) who observed such viscosity changes after continued use of the medium. The limitations of the method are such that it cannot be expected to produce great accuracy except under special conditions, but its speed and simplicity, coupled with the necessity of using only very small samples make it of great utility in the clinic and wherever rapid, approximate analyses are required.

An important and popular variation in specific gravity methods is the use of various flotation procedures. Viscosity is not a factor in these as in the falling drop, and the equipment is considerably simpler to use. Sample sizes must usually be larger than for the falling drop method, or alternatively, the equipment is correspondingly complicated (145). Single (146) or multiple (147) glass beads of known density may be floated in the sample. The most ingenious application of this principle is that of Mortensen (148) who used two glass beads in a 1 ml. calibrated tube. One bead was lighter and one heavier than the serum or plasma. On inverting the tube the beads came together, and the point of meeting was read on the tube in terms of specific gravity, or of protein composition. Flotation of a drop of sample directly in a density gradient tube was utilized by Ponder (149) and by Lowry and Hunter (150) who extended the method to determination of hemoglobin as well as serum or plasma proteins. Other variations of the specific gravity method include direct measurement by the pycnometer (132) and by Krogh's precision syringe (151).

## 2. *Refractive Index*

Protein analysis based on the effect of protein concentration on the refractive index of the solution was introduced in 1903 by Reiss (152). It was brought to greater attention by Robertson (153) in 1915. When, in 1925, physical methods of protein analysis were reviewed by Starlinger and Hartl (154), it was already recognized that the refractometric method had serious limitations. One g. of protein dissolved in 100 ml. of aqueous solution will increase the refractive index of the solvent by approximately 0.0018. Since the limiting sensitivity of the Pulfrich or Abbe refractometer is 0.0001, it is seen that with these instruments, the sensitivity of the method is definitely less than that of the specific gravity procedure. The dipping refractometer, which is somewhat more sensitive, requires also considerably more sample. The effect on the refractive index of 1 g. of a mineral salt dissolved in the same amount of solution is of the same order of magnitude as that of 1 g. of protein and may be even considerably greater. Thus, for routine analysis, the assumptions previously discussed may frequently not be valid. With proper care, the method is useful in similar degree to other physical constant methods. The conditions for application to the analysis of casein in skim milk were studied critically by Brereton and Sharp (155) in whose hands the method apparently yielded reliable results. In order to do this, the casein was first precipitated and washed, and redissolved for determination. A somewhat similar technique was illustrated for blood serum by Siebenmann (156) who measured the difference in refraction before and after heat coagulation of the proteins at pH 4.6; and for potato juice by Wolff (157) who used an interferometer with higher accuracy than the refractometer, and removed the protein by boiling and filtration. Recent studies of the refractive index method of analysis of serum proteins include those by Plötner (158) and by Sunderman (159).

## 3. *Other Physical Property Methods*

Numerous methods based on the correlation of other physical properties with protein content have been described. Some of them are so much less favorable than specific gravity or refractive index that they do not warrant extended discussion here. Others, such as electrophoresis (160, 161) and infrared absorption spectroscopy (162) are to be considered at this time as being primarily research methods until wider distribution of information and equipment is available. The interesting technique of spreading the protein on a liquid surface and measuring the area as a means of quantitative analysis was described by Hooft (163) and by Gorter and associates (164). The viscosity of formalin treated serum

was used for analysis of globulin content by Foster, Biguria and Adams (165).

#### IV. PROTEIN MASS

Determination of the actual mass or weight of the pure protein contained in the sample is obviously the objective of all protein analyses. The method of precipitating, washing, drying and weighing the protein is the most direct approach, and one which has appealed to many investigators in spite of the well known technical difficulties and uncertainties of each of these individual operations. There is no generally accepted method for drying proteins to constant weight, in spite of certain arbitrary conventions accepted by some analytical groups for particular types of analyses. Calvery (1, p. 216) states "A preparation (of protein) may be dried to constant weight at a certain temperature, but if the temperature should be increased by a few degrees, almost invariably more moisture will be lost. At present it is not possible to state when all of the moisture, which is not an integral part of the molecule, has been removed, and when some of the moisture which is obtained results from decomposition of the protein." The lyophile technique has not been used widely for drying of proteins, but appears to offer a possibility for obtaining a true dry weight (166, 117).

The accompanying major problem of establishing conditions under which the total protein or some definite and reproducible fraction of it may be quantitatively obtained, uncontaminated by fats, salts, organic matter or other non-protein material, is even more involved than the drying problem. Examination of the methods necessary to separate a single protein from a mixture of proteins (167) make it obvious that this problem is not yet resolved for practical chemical analysis. Some fractionation can be readily achieved, and many contaminants can be washed out, *e.g.*, fats are removed by acetone, alcohol or ether, simple salts by water, etc. There is little doubt that the gravimetric technique properly modified for the particular problem at hand, is adequate for some routine work, though it is not favorable from the standpoint of rapidity. It is still highly doubtful if the absolute accuracy of any of the direct methods can be made comparable to the precision, or reproducibility.

Protein mass is also estimated empirically from a large number and variety of nephelometric, turbidimetric and protein volume methods. In general, these have the advantage of being applicable to smaller samples, and being faster for routine analyses. What apparently has not always been fully appreciated is that a complete empiricism and a whole new set of possible errors are introduced with these methods. In general they

are highly dependent on reproduction of conditions and calibration against other (empirical) methods. The determinations are likely to be less reproducible than measurement of physical constants, and the interpretation of results little if any easier.

### 1. *Direct Weighing*

So many investigators have used direct weight methods as calibration for other procedures, or to check the reliability of other procedures, that a complete review of the literature will not be attempted. Many of these workers assumed that the gravimetric methods were accurate and did not consider carefully the techniques they used. In general, the removal of cholesterol and the lipides has been recognized as a critical problem and has been studied rather extensively. Acetone, or acetone-alcohol methods, which accomplish lipide separation and protein coagulation simultaneously have been rather widely used and studied (168, 169, 170, 171, 172). Methylal was recommended as a substitute for the above solvents by Delsal (173). Addis *et al.* (174) analyzed total body protein or organ protein by a variation of the gravimetric method. The carcass or whole organ was heated at pH 5 to coagulate the protein, water-soluble materials were extracted with buffer solution, fats and buffer with hot alcohol, and finally the remaining material was weighed. Non-protein materials not removed by the extraction were determined separately and subtracted.

In order to coagulate the protein or to assure that the precipitate is insoluble in the wash liquid, reagents have been used which combine with the protein to form insoluble compounds, *e.g.*, sulfosalicylic acid and mercuric chloride (175, 176), Tsuchiya's reagent (177) and formaldehyde with controlled salt concentration (178). Whatever degree of freedom from pure empiricism is gained by using the gravimetric method is certainly lost when the protein is combined with an unknown weight of reagent before weighing.

### 2. *Turbidimetric Methods*

When the sample is small, or the protein concentration low, it is convenient to form a suspension of insoluble protein or protein compound and estimate the quantity of the solid phase turbidimetrically or nephelometrically. The method is rapid, requires little sample, and can be carried out conveniently in most of the currently popular filter photometers. Moreover, the procedure is applicable to proteins which have been salted out (for fractionation) or precipitated with reagents to render them insoluble (for total protein), or even to proteins which are themselves insoluble except in the salt form, such as wheat gluten (179). The use of these procedures is certainly justified by the obvious ad-



vantages so long as the analyst is fully aware of the relatively low accuracy and a considerable lack of precision. Turbidimetric methods in general are not well adapted for accurate analysis because the light absorption is a function of the degree of dispersion of the material as well as of the concentration of dispersed material. The degree of dispersion is in turn a function of time of standing, pH, salt concentration, concentration of material, method and rate of precipitation, presence or absence of protective colloids, and other factors. Only the most rigid standardization with the most uniform samples can be expected to yield reasonable constancy of all the factors concerned. In the determination of blood protein, it is reasonable to expect a single operator to check himself by the same turbidimetric method within a few per cent. It is not unlikely that different operators working under different conditions will fail to check each other by as much as 10 to 20%. It is notable that few of the authors of this type of method claim high accuracy for them, though there are exceptions.

Typical methods based on turbidity measurements are those of Looney and Walsh (180) who precipitated total protein with sulfosalicylic acid in presence of gum ghatti, and globulin by half saturation with ammonium sulfate in presence of gum ghatti; and Mawson (181) who also used sulfosalicylic acid for total protein and 22.2% sodium sulfate to precipitate globulin. Plötner (182) recommended the turbidimetric method for serum globulin determination after salting out with one-half saturated ammonium sulfate, but condemns the use of sulfosalicylic acid as a precipitant for quantitative work. Marron (183) found errors up to 10% in using this reagent with cerebrospinal fluid. King and Haslewood (184) proposed the use of permanent suspensions of formalazine as standards for the sulfosalicylic acid method. This may have added to the method's convenience, but certainly not to its accuracy. Numerous additional reagents of no special merit have been used for precipitating protein.

### 3. *Protein Volume*

The estimation of protein mass by centrifuging the precipitated protein in a calibrated tube and estimating its volume has been seriously proposed and used (185, 186, 187). This technique has been revived several times for use with common analytical precipitates and has been rather carefully studied. Its popularity has always been short lived because it has never been sufficiently easy to standardize properly the conditions of precipitating and centrifuging to make it truly quantitative. Variations of 20% between operators would be considered reasonable, though a single operator working with rather uniform samples



might achieve a moderately impressive precision. In view of these facts, it is inconceivable that completely satisfactory results can be achieved with a method of this type used with a material such as protein. Proper calibration may produce sufficient accuracy for some purpose but there are few if any advantages of the method over other available procedures.

## V. MISCELLANEOUS METHODS

Direct titration of protein with an acid, the anions of which are bound firmly to the precipitated protein, such as metaphosphoric (188) and trichloroacetic (189) acids, offers interesting possibilities for determination of protein. Without doubt the method is more useful for determining the acid binding groups of the protein than for determining the protein itself, but under some circumstances, where the protein is of uniform composition and of calibrated acid binding capacity, it may have significant possibilities.

Determination of reagents bound to proteins as a means of protein analysis was studied for phosphomolybdic acid by Walker and Bakst (190). The bound reagent was determined colorimetrically after reduction. A somewhat similar method was described by Kerridge (191) who added a carbon suspension to the sample and precipitated the protein with trichloroacetic acid. The protein was estimated from the grayness produced by the carbon carried with the precipitate. Findley (192) found the method accurate to better than 10%, which is better than would be anticipated for this type of procedure. Glass (193) estimated mucin in various physiological fluids by virtue of its ability to bind iodine, the excess of which could be titrated back. He fractionated the mucin from other proteins first.

Immunochemical methods for protein analysis were discussed by Taylor (194) and by Kendall (195). This type of method offers considerable advantages when a single protein of a mixture must be determined, because of the high degree of specificity obtainable. It is scarcely possible to consider it as a regular chemical procedure as yet.

## VI. SEPARATION AND FRACTIONATION

*Precipitation of proteins* by "deproteinizing agents" is one of the most widely employed operations of the biochemical analyst, and one on which the available evidence is not plentiful. Probably no precipitating agent separates protein completely from all other constituents of a biological mixture. Many such agents are chosen for removal of protein in the analysis for some other constituent, on the basis of how well it also removes non-protein interference. Obviously the aim of the analyst in choosing a precipitant for protein which is to be analyzed

is the reverse, *i.e.*, to precipitate nothing which is not protein, or at least to precipitate no non-protein nitrogen. It is recognized that acid deproteinizing agents remove less non-protein nitrogen than basic reagents. Trichloroacetic acid and the Folin-Wu reagent or some of its modifications are most commonly employed. Of the non-acidic reagents, acetone and alcohol are most common. Recent work (196) with alcohol as a fractionation agent would indicate that proper application of this reagent may be expected to yield good separation of protein from non-protein materials. It is certain that the usual method of addition is not satisfactory.

The acidic deproteinization reagents were carefully studied by Hiller and Van Slyke (197), along with several non-acidic reagents. Their results indicated that 2.5% trichloroacetic acid was superior to all other reagents in separating protein from non-protein nitrogen. Increasing the concentration increased the precipitation of non-protein compounds. Both tungstic and picric acids gave higher yields of amino nitrogen, however. This raises the question as to whether the analyst wishes to include peptone and polypeptide nitrogen with protein or with non-protein nitrogen. Regardless of the decision on this question, it still remains to show what reagent is capable of yielding a clear division between the various protein degradation products at any chosen point. Hofman and Richter (198) state that the non-protein nitrogen of trichloroacetic acid filtrates is variable and affected by the dilution of the sample as well as the concentration of reagent. Peptide nitrogen was found in the filtrate at a 1 to 60 dilution. Phosphomolybdic acid was found to give essentially the same results as trichloroacetic acid. Richter (199) states that silicate filtrate of blood serum contained 24% more non-protein nitrogen than trichloroacetic acid filtrate; sulfosalicylic acid filtrate, 40% more; and phosphomolybdic acid filtrate, more than trichloroacetic acid filtrate. Dilution studies with trichloroacetic acid indicated that it did not separate the non-protein nitrogen, the separation being improved by addition of specific concentrations of certain acids. Becker and Woldan (200) compared six different precipitating agents on extracts of dried feeds and sheep feces, and found serious disagreements. When the same precipitants were used with simple systems containing no non-protein nitrogen, the results were concordant. Rondoni and Pozzi (201) found that the addition of a little hydrogen peroxide increased the amount of nitrogen precipitable by trichloroacetic acid. No denaturation could be detected, and oxidation of the protein should have produced the opposite effect. All of these findings might be considered of little significance if it were not for the fact that there appear to be few complete and careful studies on this important

question. Nearly all the studies of deproteinization methods are designed to find reagents which remove other particular materials whose presence may prevent accurate analysis of some constituent other than protein.

Metaphosphoric acid has received less attention than many other acid reagents which precipitate proteins. This may be due in part to the instability of the reagent, which makes uncertain its exact composition. A useful recent study of this matter was made by Briggs (202). Further work on its effect in precipitating non-protein nitrogen should be performed in view of the uncertainty of the older work. Neuberg and Strauss (203) have proposed perchloric acid as an improved reagent as compared with trichloroacetic acid. Apparently, no studies are available which indicate the extent of precipitation by this reagent of nitrogen compounds other than protein.

*Fractionation of proteins* is too large a field to be completely reviewed here, but certain facts stand out clearly. The distinction between different classes of proteins, e.g., albumins and globulins, was for a long time made completely on the basis of solubility in water, salt solutions and other solvents. The inadequacy of such a method of classification was pointed out by Block<sup>1</sup>, who attempted to introduce chemical factors into it as well. Butler and coworkers (204) had previously shown that albumin and globulin separation by neutral salts was not sharp, and that there was much overlapping of the fractions. Dulière (205) found poor agreement between the sodium sulfate method of Howe (206) and the ammonium sulfate method, while Macheboeuf (207) concluded from a comparative study of several fractionation methods, that the definitions of the protein fractions were indefinite unless the method of separation was specified.

It is now apparent from electrophoresis and ultracentrifuge measurements, correlated with separation by neutral salts (167, 208, 209, 210, 211), that the latter are incapable by any known means of accurately fractionating protein mixtures without complicating the operations to a point at which they are of little use as practical analytical procedures. Thus, Dole (212) found that the albumin-globulin ratio measured electrophoretically was about 2/3 of the ratio found by chemical fractionation, an observation which was also checked by Pillemer and Hutchinson (196) by use of a methanol fractionation which was checked against electrophoresis.

It appears that the protein analyst must now accept the fact that the simple salt fractionations are obsolete and undependable, and that this

<sup>1</sup> Ref. (1), Chapter VII, The Chemical Constitution of the Proteins.

central problem of protein determination must be solved by other methods. That the problem is not insoluble is indicated by investigations of several new methods of fractionation<sup>2</sup>, such as that of Pillemer and Hutchinson (196) who were able by a simple fractionation with methanol to check electrophoretic methods for albumin and globulin fractions to  $\pm 5\%$  on normal sera and to 5 — 10% on abnormal sera. Other studies of alcohol fractionations have been published by Cohn (167) and by Taylor and Keys (213)

It is considered that the most fundamental problems of protein analysis, particularly as it is used for research purposes requiring absolute results, are the problems of precipitating proteins from other constituents and fractionating the separated proteins. To be satisfactory as a general analytical method, the procedure should require a minimum of time and technical complexity, and at the same time give results comparable with those now obtainable by use of electrophoresis and the ultracentrifuge. When this may be accomplished, the problem of finally determining the separated protein will be of secondary importance. In the meantime, analyses of reasonable reproducibility may be made by several general procedures which are adequate for many routine uses. The Kjeldahl nitrogen determination, the biuret reaction and the specific gravity methods particularly have shown themselves to be practically useful despite their many limitations. If the analyst is fully aware of the inherent deficiencies of these methods, he may obtain continued useful service from them. At the same time newer developments, many of them underway now, should eventually relegate all present methods for accurate analysis to obsolescence.

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<sup>2</sup> It is believed that there is much as yet unpublished work on fractionation of serum proteins particularly by alcohol methods, which is not yet available to the author, and which may go far to resolve this problem for analytical purposes.

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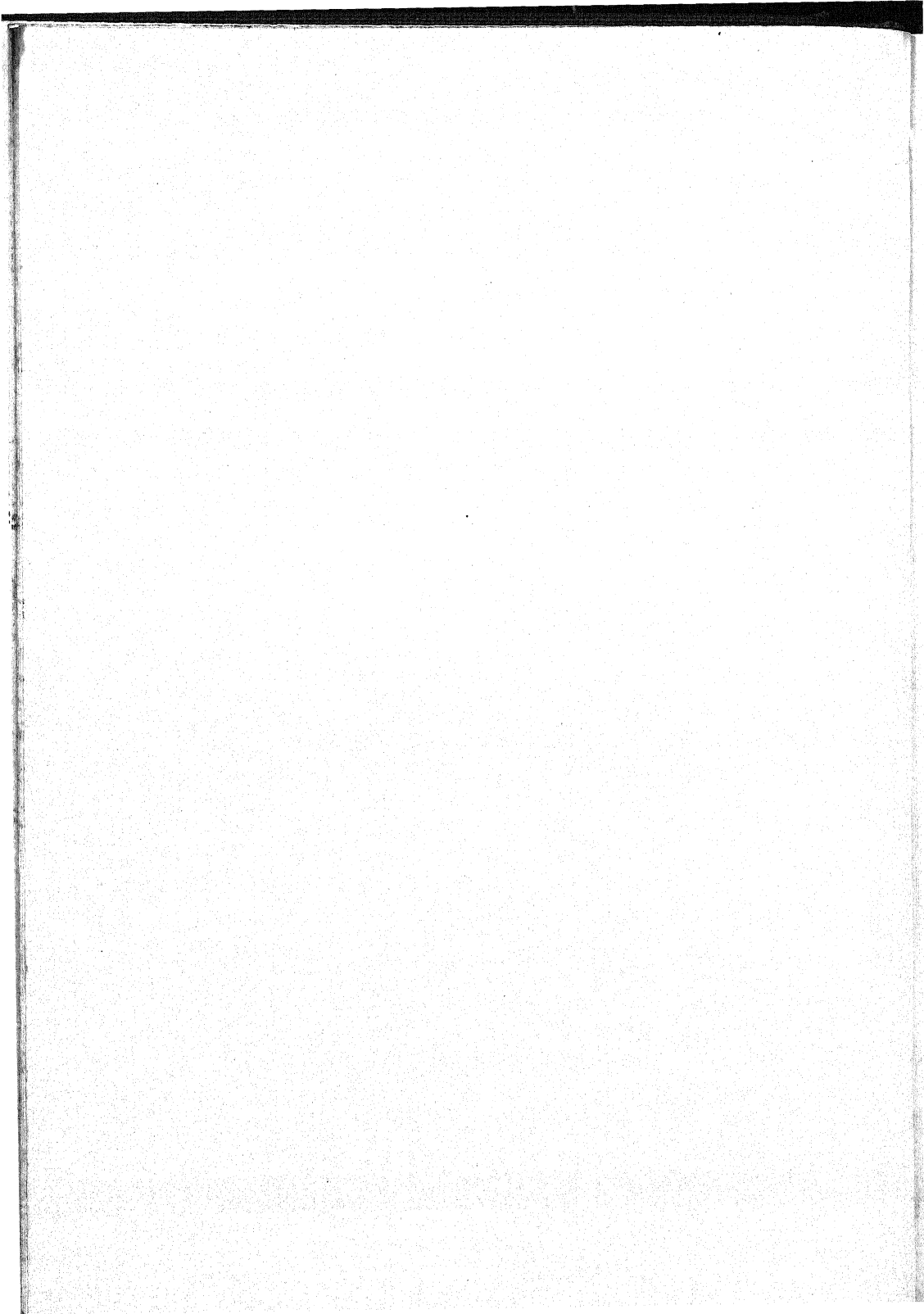
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# Reactions of Native Proteins with Chemical Reagents

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## I. INTRODUCTORY REMARKS

Interest in the modification of proteins by means of chemical reagents has risen sharply in recent years and has paralleled the appearance of fairly convincing proof of the protein nature of such biologically important materials as antigens, enzymes, toxins, some hormones, and viruses. This interest naturally falls into two classes: the theoretical and the practical.

Theoretical interest is directed toward determining the nature of the structures which are responsible for the distinctive biological action of certain proteins. Examples may be found in the numerous researches on the structures of antigens (1), hormones (2, 3), pepsin (4-8), and tobacco mosaic virus (9). However, this work has only just begun. The structure of no protein is well understood.

The practical interest is for the most part focussed on the modification of the undesirable properties of certain proteins so that they may be used medicinally. One can think of numerous examples, such as the preparation of toxoids and vaccines.<sup>1</sup>

The work in a number of laboratories (10-13) on the conjugation of carcinogens to proteins for the purpose of producing antisera bridges both classes of interest.

In this review those chemical reactions are considered which can be used to modify native proteins under such mild conditions that little or no denaturation may be expected. It is of considerable importance that no denaturation take place, for if denaturation assumes appreciable proportions the system is very complicated and no reliable interpretation can be made of the results.

Very few workers have established the fact that a chemical reaction has been carried through involving specific groups of a protein without denaturing the protein in the process. In many instances denaturation has undoubtedly preceded, or accompanied, the specific chemical reaction. It has been reported, for example (17), that serum proteins lose their immunological specificity on standing at pH 11.0. Denaturation is probably involved in this change, yet coupling of diazonium compounds to serum proteins has often been carried out at pH 11.0 or even more alkaline. In such cases, not only is the chemistry difficult to interpret because of the effect of denaturation on the specific reaction, but the serology as well.

The best proof that the change in the protein results from the chemi-

<sup>1</sup>Toxoids and certain vaccines are detoxified or inactivated toxins, viruses, or bacteria, whose antigenic specificity is qualitatively retained and will, therefore, as antigens, elicit the formation of a protecting antiserum to the original active agent.

cal reaction under consideration and not from the non-specific denaturation is furnished by reversing the chemical reaction followed by a demonstration that the recovered protein has the same properties as the original unaltered material. In a number of instances (4, 125, 32, 54, 72, 199, 26) removal of the bound reagent residues has brought about a recovery of biological activity. However, many of the chemical reactions are not reversible, at least under such conditions that the original protein would be expected to remain native.

A definition of denaturation, or of a denatured protein, would seem to be a prerequisite by which to judge whether the observed change in the protein had been brought about by the specific reagent, by denaturation, or both. However, there is at present no single criterion which covers all cases. The usual one based on the insolubility of the denatured form in dilute salt solutions near the isoelectric region is not rigidly applicable, for many of the chemical derivatives are much less soluble under these conditions than the original protein, yet are probably native, since they possess biological activity (4, 7, 14, 15, 16). It is desirable, therefore, to use many criteria and to compare the protein derivative with the original protein by as many of them as is feasible. Solubility, crystallinity, biological activity, digestibility by proteases, size and shape as indicated by the sedimentation and diffusion constants, viscosity, electrophoretic mobility, birefringence, absorption spectrum, and SH groups are characteristic properties of proteins which in many, but not all, instances, change when they are denatured (18, 19). The extent of these changes depends to a considerable degree on the protein and the method of denaturation. Undoubtedly secondary destruction is responsible for some of the changes in properties which have been noted. It is difficult to distinguish, from the evidence available—except in the relatively few cases of reversible denaturation—between the primary denaturation and the secondary destruction and their separate effects on the above properties.

Since many workers have not examined their reaction products by these criteria, a simple broad criterion was arbitrarily chosen for purposes of examining the literature for this review. It was assumed that denaturation was not responsible for the observed changes if the control samples (in which the active specific reagent was omitted) showed no appreciable change in properties during the experiment, or if it were known that the protein is stable under the conditions used.

A few instances have been discussed in the present review in which denaturation undoubtedly accompanied the chemical reaction under examination. In these cases either no other evidence was available or a particularly interesting point was being discussed. Some of the papers

cited deal with proteins other than those usually termed native proteins, but it was felt that the work was carried out under conditions which would make possible application of the results, at least qualitatively, to strictly native proteins.

In making studies of a specific chemical reaction it is obviously important to start with a relatively pure protein preparation, particularly if analyses of specific groups are to be made. However, even if the starting preparation is relatively homogeneous, the product after treatment with the reagent may be very inhomogeneous due to the different types, as well as large numbers of groups in proteins capable of reacting with many of the reagents. Very few chemical reagents are group specific; and unless the reaction is carried to completion, *i.e.*, to saturation of the protein by the reactant, non-uniformity of reaction products might be expected. In general when more than one type of protein group reacts with a reagent, conditions may be found which favor reaction with one type in preference to others. A careful study of such variables as temperature, time, and pH has in many instances permitted a choice of conditions which render the reagent more nearly group specific. For many reactions these conditions are not known, and as a consequence a mixture of chemically different derivatives might be expected. With such products chemical analyses have a limited value unless these preparations are fractionated or accompanied by some evidence bearing on their homogeneity. Measurements of the physical and chemical properties mentioned earlier in the discussion of denaturation are also useful as evidence bearing on the homogeneity of modified proteins.

The reviewer has been surprised at the high degree of homogeneity of certain reaction mixtures. When pepsin was treated with ketene (9) or iodine (7) under mild and controlled conditions the bulk of the reaction mixture was relatively uniform, as shown by fractionation procedures designed to detect the presence of products with extremely different properties. This result may be interpreted as indicating that most of the groups of the type which react first, *i.e.*, at the highest rate, are saturated before those of the next type have reacted appreciably.

A correlation of the number of specific protein groups modified with the number of reagent residues introduced into the protein is another desirable feature of any study of the type covered by this review. In the case of oxidation or reduction reactions in which no reagent residues are attached to the protein the stoichiometry of the reaction provides equivalent information.

Most of the chemical reactions to be considered are non-ionic and involve the formation of relatively stable covalent linkages.

The interesting and important field of chemical reactions in non-

aqueous solvents has not been discussed in this review. This field of investigation should expand rapidly as a result of improved methods of drying proteins at low temperatures in such a way that the native structure is retained.

## II. CHEMICALLY REACTIVE STRUCTURES OF PROTEINS

Native proteins, in general, contain structures which are usually associated with particular amino acids. Details concerning the characteristics and methods of estimation of these structures or groups may be found in the following references (18, 19, 20), but a few words may give a general picture of the variety and nature of these groups and methods of quantitatively estimating their presence.

Whenever possible methods which are applicable to intact proteins have been noted but for certain groups, *e.g.*, aliphatic hydroxyl groups, no such methods exist. In such cases the methods indicated are performed on protein hydrolyzates.

The conditions usually employed in the measurement of certain protein groups may denature the protein in the process of measurement, and since it has been shown (73, 168) that the measurable number of some protein groups increases with denaturation such measurements must be interpreted with great care. To the above difficulties should be added the fact that for a single type group such as SH, the number measured varies with the method (144, 47).

1. Carboxyl groups (COOH) of proteins are usually associated with the dicarboxylic amino acids, aspartic and glutamic. This association has not always withstood quantitative scrutiny (18, page 480). With  $\beta$ -lactoglobulin the correlation is good (21), although the evidence suggests also the presence of about four  $\alpha$ -carboxyl groups per molecule.

No specific method for determining carboxyl groups in the intact protein is available. The reviewer has found it convenient to estimate them from the titration equivalent between pH 5.5 or pH 6.0 and pH 2.0, depending on the protein and its histidine content. When the histidine content was high, as in hemoglobin, the starting point was set at pH 5.5. This method is open to criticism because of this overlapping of the titration regions of the two types of groups, but the uncertainty of the value obtained is more than offset by the usefulness of the method.

2. Amino groups ( $\text{NH}_2$ ) have usually been assigned to the  $\epsilon$ -nitrogen of lysine in proteins (22) but investigations over the past decade have shown that in many instances  $\alpha$ -amino groups of other amino acids are free. Thus in insulin (226), egg albumin (237), cytochrome C(383), and  $\beta$ -lactoglobulin (244, 21), the amino nitrogen of the intact protein was greater than expected from the lysine content. The amino groups of phenylalanine has been found free in insulin (23, 374) and in casein, globin, and ovalbumin (375) while that of glycine was demonstrated in insulin (374). Amino groups are usually determined by



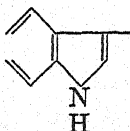
Van Slyke's nitrous acid procedure (24), by the formol titration (25), or by use of the ninhydrin reaction (26). These three methods do not always give the same value. The nitrous acid method probably gives the best value for the total amino nitrogen. The  $\alpha$ -amino group may be expected to be titrated between pH 7.0 and 9.0, whereas that of the  $\epsilon$ -amino nitrogen of lysine is nearer pH 8.5-11.5.

### 3. The phenolic group



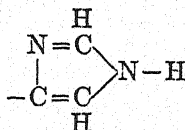
belongs to tyrosine and may be determined by the quantitative Millon-Lugg method (27) or by means of Folin's phenol reagent at pH 8.0 (28). It reacts with alkali (when free) between pH 8.5-11.5.

### 4. The indole imino group



arises from tryptophan and may be determined colorimetrically by Shaw and MacFarlane's modification of the glyoxylic method (29).

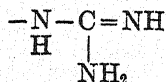
### 5. The imidazole NH group



from histidine has been determined colorimetrically by Pauly's method modified by Jorpes (30). It is titratable and has a pK of about 6.7.

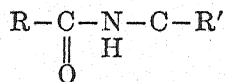
### 6. The SH groups of proteins belong to cysteine and can be estimated in several ways (32, 33, 34, 85). The pK of the SH group is about 10.0. It is titrated over the range where tyrosine phenol and $\epsilon$ NH<sub>2</sub> of lysine are titrated, pH 8.5-11.5.

### 7. The guanidino structure



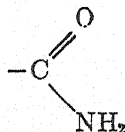
belongs to arginine. It may be determined with the aid of the Sakaguchi reaction, modified by Weber (35). The group is strongly basic, reacting between pH 10.0 and 13.0.

8. Disulfide linkages (-S-S-) belong to cystine and they are readily determined after preliminary reduction to SH groups (31). They do not bind acid or alkali.
9. The aliphatic hydroxyl groups of proteins are associated with the amino acids, serine, threonine, and hydroxyproline, with an occasional appearance of hydroxylysine and possibly hydroxyglutamic acid. The periodate method of Nicolet and Shinn (36) appears to be the best procedure for the estimation of these amino acids. Hydroxyproline may be determined colorimetrically (37, 38).
10. Methyl sulfide ( $\text{CH}_3\text{-S}$ ) groups arise from methionine and may be determined by the method of Baernstein (39) or colorimetrically (40).
11. Phenyl radicals ( $\text{C}_6\text{H}_5$ ) from phenylalanine are determined by the Kapeller-Adler method as modified by Kuhn and Desnuelle (41).
12. The peptide linkage



may be estimated by the biuret reaction which has been made quantitative by several investigators (42-44).

13. Amides



are associated with glutamine, the amide of glutamic acid, and asparagine, the amide of aspartic acid. A quantitative estimation of such groups is obtained from ammonia analyses after acid hydrolysis (45).

### III. REACTIONS

#### 1. Oxidation

The oxidation of proteins can be carried to any degree desired by choosing the appropriate reagent and conditions. Oxidation of sulfur groupings of proteins has received the most attention of investigators but tyrosine and tryptophan are also oxidized under certain conditions. The SH groups, when present, appear to be the first to be oxidized. However, not all native proteins have free SH groups. To complicate the picture further, the free SH groups in native proteins differ markedly in their reactivity toward the various reagents. Anson has shown (34), for instance, that native egg albumin has no free SH groups when tested with nitroprusside, ferricyanide, or porphyrindin; but dilute iodine

oxidizes all the groups of native egg albumin which become SH groups, by all tests, when the protein is denatured.<sup>2</sup>

Hellerman and his associates (32, 46, 47) have demonstrated in some very carefully planned and analyzed experiments that within a given protein the SH groups differ markedly in their reactivity. Thus the "a" type SH groups of crystalline urease, which respond positively to the nitroprusside test, were oxidized by dilute ferricyanide, dilute iodosobenzoate, or by porphyrindin, or made to react with iodoacetamid without effect on the enzymatic activity. Further oxidation of "b" type SH groups by strong iodosobenzoate or iodine, or reaction with *p* chloromercuri-benzoate or iodoacetamid, resulted in complete inactivation. The inactivation was completely reversed by  $H_2S$  or cysteine. In addition to the "a" and "b" SH groups, they found that after denaturation of urease with guanidine hydrochloride there were additional SH groups which were detected by all of the reagents. These workers indicate (32) that there is one gram equivalent of "a" type SH group, one of "b" type, and two to three of the last mentioned type SH groups for every 21,300 g. of urease. With a molecular weight of 483,000 there are then about twenty-three "a" type, twenty-three "b" type, and forty-five to seventy other SH groups per mole urease. It was also noted that strong iodosobenzoate failed to oxidize tyrosine, tryptophan, serine or methionine under similar conditions.

Sizer has been carrying out interesting oxidative studies on the enzymes urease (48), invertase (49), bovine phosphatase (50), and crystalline chymotrypsin (51). He has followed the inactivation of these enzymes with dilute reagents of varying oxidative potentials and finds that the enzymes are stable up to a certain potential beyond which they are rapidly inactivated. Urease activity is a continuous function of oxidation reduction potential. The activity is rapidly lost at  $E_h = -260$  mv. and  $+540$  mv. with the  $E_h$  of optimal stability at  $+150$  mv. Invertase is stable for 10 minutes at pH 4.6 in solution of potential  $E_h = +600$  mv., but at higher potentials there is a sharp drop in activity to zero at approximately 1000 mv. Chymotrypsin (51) is stable for 5 minutes at  $37^\circ C$ . at potentials up to  $+500$  mv. but its activity is zero at 650-700 mv. The inactivation can be partially reversed for the phosphatase (50) and chymotrypsin (51) by dialysis or addition of certain reducing agents, such as ferrocyanide. Sizer suggests that oxidation takes place at the tyrosine, since the ultraviolet absorption spectrum shift is the same as that noted for free tyrosine treated similarly (50). Upwards of a dozen different oxidizing agents have been used

<sup>2</sup>For a complete discussion of this work, the reader is referred to the article by M. L. Anson in the *Advances in Protein Chemistry*, vol. 2 pp. 361-386.

which, as Sizer points out, rules out the possibility that the effects were produced by a toxic reaction, or some individual idiosyncrasy of one or more agents.

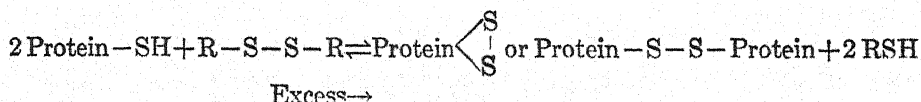
It should be possible to determine the potential at which known peptide or protein structures are oxidized and to use this in the identification of groupings oxidized in particular proteins.

Many protein systems besides those discussed have been subjected to oxidative studies. Some of these are carbonic anhydrase (52), papain, and catheptic-like enzymes (53-57), invertase (58), succinic dehydrogenase (59), triosephosphate dehydrogenase (60), glycerol oxidase (61), and scarlet fever toxin (62).

Reviews by Hellerman (63, 47) and by Bersin (64) cover many cases not mentioned here.

The various types of agents used in the oxidation of groups in native proteins will now be discussed individually

a. *-S-S- Compounds.* Hopkins (65) showed that free SH groups of soluble muscle protein, which give a positive nitroprusside test, are oxidized at pH 7.6-8.0 with solutions of the oxidized form (-S-S-) of glutathione. The reaction could be reversed by treating the oxidized protein with an excess of reduced (SH) glutathione. The following equation illustrates this reversible equilibrium:



No reaction occurred when the solution was at pH 3.0-4.5.

Mirsky and Anson, in a series of papers (31, 66, 67) have extended these studies and developed quantitative methods for the estimation of SH groups. They have found that in general the reactivity of the "reducing groups" of proteins (those which reduce the oxidizing agent and in turn are oxidized) increases with pH, temperature, and denaturation. Using an excess of cystine at pH 9.5, the SH groups of native hemoglobin were oxidized (67). The absorption spectrum of this oxidized hemoglobin was similar to that of native hemoglobin, indicating that no denaturation had occurred. Balls and Lineweaver (53) have inactivated crystalline papain by oxidizing the SH groups of this protein with cystine.

Oxidation by -S-S- compounds appears to be one of the few specific chemical reactions of proteins; for, so far as is known, only SH groups are affected and they are oxidized to -S-S- linkages. The oxidant must be present in considerable excess, since the reaction is reversible and the concentration of -S-S- oxidant determines the extent of the reaction.

b. *Iodine.* Very dilute iodine in potassium iodide solution inactivated

papain (54, 63) and urease (46) by oxidizing SH groups, and this inactivation was reversed to a considerable degree by  $\text{H}_2\text{S}$  or cysteine. Since these earlier papers a thorough study has been made of the use of iodine to estimate SH groups of proteins in general (68, 69). It was shown that SH groups, or their precursors which became SH groups after denaturation, were oxidized by dilute iodine at either pH 3.2 or pH 6.8. At pH 6.8 the oxidation was carried out in the presence of molar potassium iodide at  $0^\circ\text{C}$ . which depressed the reaction between iodine and the tyrosine of proteins. Under these conditions, all the SH groups (and apparently no other groups) of native egg albumin were oxidized stoichiometrically (68). Tobacco mosaic virus on the other hand required about 2.5 times the stoichiometric amount (68). At pH 3.2 iodine reacts with pure tryptophan but chymotrypsinogen, which is rich in tryptophan failed to reduce any iodine. At this pH an excess of iodine will carry the oxidation of SH groups beyond the -S-S- stage but, oddly enough, it does not oxidize the -S-S- groups at least of cystine or of insulin (69).

Iodine at pH 4.5 has been used to oxidize SH groups of  $\beta$ -amylase (72). It has been claimed (70) that phosphate ion accelerates the oxidation of proteins by iodine at pH 5.9, but in this work there was no differentiation between oxidation and substitution.

Iodine in molar potassium iodide appears to be a useful agent for the oxidation of protein SH groups since it reacts fairly rapidly in dilute solution; it can be estimated precisely, the conditions of the reaction are those of maximum stability of most proteins, and thus far it has not failed to oxidize all the SH groups in the native protein

*c. Ferricyanide.* Native hemoglobin SH groups have been oxidized by ferricyanide in neutral or slightly alkaline solution (67, 71). It was found that the number of SH groups oxidized increased with alkalinity (67). An examination of the absorption spectrum indicated that no denaturation occurred during oxidation by ferricyanide, even at pH 9.6. Papain (54) and  $\beta$ -amylase (72) are reversibly inactivated by oxidation of their SH groups with dilute ferricyanide near pH 5.0. In the latter case (72)  $\text{Cu}^{++}$  was required.

Some protein groups other than SH are oxidized by ferricyanide (73). This was indicated by the increased quantity of ferricyanide reduced as the concentration of ferricyanide was increased. Since tyrosine and tryptophan, but not histidine, reduce ferricyanide it has been suggested that it is these two amino acids in proteins that are oxidized. This was given some support by the fact that gelatin which is devoid of tyrosine and tryptophan failed to reduce ferricyanide, while zein which contains tyrosine, but not tryptophan, reacted rapidly. However, native egg

albumin, which has SH groups and both tyrosine and tryptophan, failed to reduce ferricyanide (69). In the absence of evidence to the contrary it may be presumed that tyrosine in some proteins may be oxidized by ferricyanide.

As indicated earlier, SH groups of different proteins vary in their reactivity. With native egg albumin ferricyanide is not reduced at all, even though the protein contains SH groups and tyrosine. Thus, not only SH groups, but also tyrosine phenol groups, differ in their reactivity.<sup>3</sup>

Ferricyanide has the advantage of reacting quite rapidly with protein SH groups, under biological conditions. It is stable, and readily available. The ferrocyanide formed can be easily and precisely determined as Prussian blue in extremely low concentrations. However, this reagent appears to be non-specific in some instances and completely ineffective in others.

*d. Hydrogen Peroxide.* Hydrogen peroxide has been used with varying results. Hellerman found it had no more effect on urease than uncatalyzed aeration (46). Inactivation of insulin (76) was carried out with 15%  $\text{H}_2\text{O}_2$  at room temperature, while tobacco mosaic virus (77) was inactivated with 5%. This action on the virus was carried out at pH 7.0 and resulted in a 60% drop in amino nitrogen. Papain (78) was inactivated with  $M/500$   $\text{H}_2\text{O}_2$  in 5 minutes at 40°C. These results show considerable differences in reactivity, even after due allowance is made for the differences in conditions.

Freudenberg and coworkers (79, 80) have carried out some interesting experiments with inactive insulin in which the -S-S- linkages have been reduced to SH groups. It appeared that in reversing the reaction by oxidation it might be difficult to oxidize (dehydrogenate) two protein SH groups to an -S-S- linkage; but if an excess of some other SH compound were added, an SH group of the protein and one from the introduced compound might be expected to be joined and an -S-S- linkage formed more easily. This would not result in the original structure but it would bring about the conversion of protein SH groups to -S-S- linkages. The non-protein SH compounds also depressed the possible oxidation of insulin SH groups to higher oxidative levels. These workers added an excess of cysteine or reduced glutathione to the reduced insulin and then an excess of  $\text{H}_2\text{O}_2$ . The extent of reactivation of the hormone varied considerably but in many instances reversal was apparently obtained.

<sup>3</sup> Egg albumin may be an abnormal protein in this respect. It should be pointed out that, whereas most of the tyrosine phenol groups of egg albumin could not be detected by titrimetric (74) nor spectrophotometric (75) methods, they apparently react normally with the Folin's phenol reagent at pH 8.0 (5).



ion is probably not due to its combination with proteins, but rather to its catalytic accelerating effect on the oxidation. Pillemer, Eckers and associates have oxidized crystalline urease (87), keratins (88), and lens proteins (89) by passing a stream of air through a solution or suspension of these proteins at pH 8.0-9.0 in the presence of small quantities of  $\text{Cu}_2\text{O}$ . White and Stern (90) noted a decrease in the SH groups of reduced insulin when an equivalent amount of oxygen was absorbed in the presence of  $\text{Cu}^{++}$  or iron.

Native proteins have also been oxidized photochemically by visible light in the presence of sensitizers, such as hematoporphyrin or eosin. It was shown many years ago by Harris (378) that egg albumin and edestin, but not gelatin, absorb oxygen when exposed to light in the presence of hematoporphyrin. These facts suggested that the aromatic amino acids were being oxidized. Similar results with solutions of tyrosine and tryptophan tended to confirm this. These results have been confirmed (359, 360, 361), using only visible light, and it was shown that histidine could also be oxidized by this procedure. Purines (326) likewise were oxidized but aliphatic compounds were not. Work along this line has recently been extended (363), the techniques simplified, and the effect of many of the variables determined (364).

A paper on oxidation of proteins has recently appeared which deserves careful consideration and further investigation. Sizer (365) has reported that the tyrosine in a number of proteins was oxidized at pH 7.3 and  $37^\circ\text{C}$ . by crude or purified tyrosinase. He found that oxygen was consumed, the Millon's reaction for tyrosine became weaker, and the ultraviolet absorption curve changed when solutions of commercially prepared crystalline pepsin, crystalline trypsin, crystalline chymotrypsin, casein, and insulin were acted upon by mushroom tyrosinase. However, no loss in activity was observed for the three proteases, there was no change in the phenol color value using Folin's reagent, and only 10-20% of the total tyrosine content of these proteins was oxidized. When highly purified human and bovine serum proteins were similarly treated no oxidation was detected. The writer does not mention any attempt to further purify the protease preparations which, in spite of their crystallinity, probably contain considerable quantities of split products. These proteases also autolyze at an appreciable rate under the conditions used, liberating tyrosine or peptides containing tyrosine. If Sizer's conclusions can be confirmed, his work is of considerable importance, for the reviewer does not know of any well-established case of non-proteolytic enzymes acting directly upon native proteins.

*h. Folin's Phenol Reagent.* The reviewer (5) has applied Folin's phenol reagent (91), a phosphotungstic-phosphomolybdic acid complex,



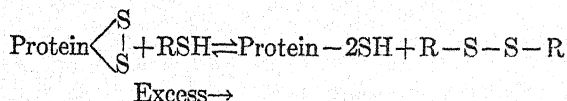
to proteins at pH 8.0, under conditions of maximum stability for most proteins. Since the reagent is reduced, as evidenced by development of a blue color, the proteins must be oxidized. The amino acids tyrosine, tryptophan, and cysteine are oxidized by this reagent at pH 8. Although this reagent has been used only for analytical purposes, it should also be considered and used as an agent for oxidizing native proteins. Under the conditions described it appears to react with about 59% of the total tyrosine plus tryptophan present in certain proteins (5, 92, 93).

*i. Miscellaneous Oxidants.* The following agents have also been used and may be of interest: chlor indophenol (94, 95), permanganates (96, 97), ascorbic acid plus cupric ion (98), hypochlorite (99-101), perbenzoic acid, (102, 103), HgO (102), benzoquinone (78, 104), selenite (78), and nitrous acid (72).

## 2. Reduction

At present no groups other than -S-S- are known to be reduced except under conditions which denature the proteins. It may be that other groups can be and are reduced but are not detected. With certain reducing agents SH compounds in particular, precise analytical methods (31) make it possible to measure quantitatively the amount of reducing agent consumed and to correlate this with the reduction of the protein groups and thus show whether only -S-S- groups are affected.

*a. SH Compounds.* In the section on oxidation, the oxidation-reduction system  $-S-S- \rightleftharpoons SH$  was discussed, in particular the use of -S-S- compounds for the oxidation of native protein SH groups. In a sense the reverse reaction



is now to be described. Hopkins (65) found that reduction of protein -S-S- groups could be brought about in slightly alkaline solution by the addition of any of several SH compounds, such as glutathione, cysteine, or thioglycolic acid. This reaction has been extended to many native proteins, and many sulfur compounds have been employed. Thus insulin (79, 80, 90, 103-107), lens proteins (108), crystalline urease (87, 107), crystalline rattlesnake toxin (109), papain (107), and certain cathepsins have been studied in this way. Reduced glutathione (65, 79, 80, 90, 110), cysteine (90, 106-115), thioglycolate (88, 106-108, 115),  $\alpha$ -thiolactate (106),  $\text{H}_2\text{S}$  (76, 87, 116), and thiosalicylate (104), are some of the SH reducing agents used for this purpose. Thioglyoxalines, thiol-histidine, and ergothionine (104) were also used but without any measurable effect.

Although reductions have been carried out in solutions of extreme pH (107), in most instances the medium was neutral or slightly alkaline. The various SH compounds differ markedly in their effectiveness. It has been reported (115) that thioglycolate was 50 times as effective in reducing the lactogenic hormone as was cysteine.

Use of SH compounds to protect the viruses of Rous sarcoma (117), herpes (118), tomato spotted wilt (119), and eastern equine encephalomyelitis (120) against spontaneous inactivation is of interest in this connection.

b. *Cyanides*. Walker (121) has reported that the disulfides of some proteins could be reduced by alkaline cyanide, as shown by a positive nitroprusside test. This method of reduction has also been used on the following native proteins: insulin (122, 123), certain other hormones (110, 116), urease (87), and lens proteins (108). Walker pointed out that in some instances certain groups are not detectable in the native protein but became so upon denaturation. Cyanide is probably a weaker reducing agent than cysteine, since the former did not affect the action of the latter (106) nor did it cause further reduction.

c. *Catalytic Hydrogenation*. In this category the reviewer has placed those cases in which so-called "nascent" hydrogen was used, as well as hydrogenation in the presence of surface catalysts. Some of the methods are difficult to evaluate and will, therefore, be noted without comment.

Insulin, one of the few proteins able to remain native in strong acid, has been reduced with zinc and hydrochloric acid (102). Parallel with the destruction of activity there was a drop in specific optical rotation  $[\alpha]$  from  $-70^\circ$  to  $-24^\circ$ . The authors point out that cystine, on being reduced to cysteine, changes its  $[\alpha]$  from  $-224^\circ$  to  $-13^\circ$ . Allen and Murlin (124) observed reversible inactivation of insulin by tin and dilute acid. They also claimed similar results by merely exposing the hormone to commercial hydrogen.

2% sodium amalgam, activated magnesium, and hydrogenation in the presence of platinum have all been employed by Freudenberg and his collaborators (76).

Harington and Neuberger (125) have used hydrogen in the presence of palladium on barium sulfate to reduce the iodine of iodinated insulin. The pH was fixed by the presence of 50% pyridine. Two thirds of the iodine was removed from the iodinated hormone with a corresponding recovery of hormonal activity.

It seems to the reviewer that careful use of sodium, magnesium, or aluminum amalgams in strongly buffered solutions might lead to fruitful results in experiments where it is desirable to go beyond the reduction of -S-S- groups.

d. *Miscellaneous.* There are a few miscellaneous procedures employing the use of  $\text{SO}_3$  (31, 76),  $\text{SO}_2$  (116) and ascorbic acid (110).

### 3. Alkylation

a. *Methylation.* This reaction may be performed with dimethyl sulfate  $(\text{CH}_3)_2\text{SO}_4$ , methyl iodide, methyl bromide, or by use of diazomethane  $(\text{CH}_2\text{N}_2)$ . The first three of these agents act in neutral or slightly alkaline solutions, pH 6.0-9.0, while the diazomethane reacts over a much greater range of pH. In general, these agents act on carboxyl, amino, and tyrosine phenol groups of proteins, but, as will be brought out later, other groups apparently react to some degree. At present our knowledge of the action of these agents on proteins is extremely meager and does not permit more than a statement of the experimental results. It is quite possible that further study will reveal that proper choice of conditions is all that is required for selective action of these agents.

The ease with which the methyl groups are hydrolyzed from a methylated protein varies enormously with the nature of the group to which the methyl radical is attached. Methyl esters may be expected to hydrolyze readily in very weak alkali (pH 11.0-12.) while the ethers resulting from methylation of the tyrosine phenol will require high temperature and strong acid. N-methyl groups will split with more or less difficulty depending on the structures in close proximity to the nitrogen. There are standard methods of determining the various N- $\text{CH}_3$  groups.

Edlbacher (126, 127) studied the action of dimethyl sulfate on edestin and other proteins and concluded that mainly lysine  $\text{NH}_2$  groups reacted. Those proteins which contained no lysine failed to react. He also isolated methyl  $\epsilon$ -amino lysine from methylated casein (128). Haurowitz (129) has recently treated crystalline horse hemoglobin and egg albumin with dimethylsulfate and obtained as an upper limit 6.5% and 3.9%, respectively, of  $\text{OCH}_3$  groups. This is 142 and 54 methoxyl groups per mole protein and represents a large proportion of the total ionizable groups for these two proteins (18).

Charles and Scott (130) using methyl iodide on crystalline insulin at  $56^\circ\text{C}$ . observed a 95% inactivation of the hormone. On subsequent treatment at  $0^\circ\text{C}$ . for 12 hours in  $N/10$  NaOH they obtained a 30% recovery of hormonal activity. It may be surmised that most N- $\text{CH}_3$  groups, and certainly the methyl ether linkages to tyrosine, would not be ruptured by this action. Methyl esters, on the other hand, would be expected to split under just these conditions. More recently Jensen, Evans, Pennington and Schock (131) have noted a decrease in the cystine content, as well as the amino nitrogen of crystalline insulin on treatment with diazo-

methane. With methyl iodide they observed a drop in the cystine, but no change in the amino nitrogen.

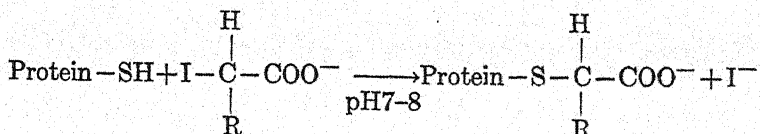
In some preliminary experiments several years ago, the reviewer (132) treated aqueous crystalline pepsin solutions at pH 4.8 with increasing amounts of diazomethane. The peptic activity decreased to 10% with the introduction of 15 methoxyl groups. This agent does not react nearly so rapidly with carboxylate ions as with the un-ionized carboxyl group. More experiments are necessary in the present case to decide whether at pH 4.8, where most of the carboxyl groups are ionized, methylation of these groups takes place. Reaction could take place with the smaller fraction of un-ionized groups in equilibrium with the ionized at this pH and the reaction could go to completion, *i.e.*, all the carboxyl groups could be methylated. No estimations were made of  $\text{N-CH}_3$  groups. Rutherford, Patterson and Harris (133) have obtained similar results and have noted a 75% drop in tyrosine content. These results on pepsin were quite similar to the results obtained when silk fibroin was similarly methylated. In this work they showed that the alkali-stable methoxyl groups agreed quantitatively with the decrease in tyrosine content (Lugg's method) and that this represented at least two thirds of the total number of methoxyl groups. They reasoned that the alkali-labile methyl radicals were probably on carboxyl groups.

Very recently Fraenkel-Conrat and Olcott (137) have apparently succeeded in specifically methylating carboxyl groups of proteins by suspending the dry protein in methyl alcohol and catalyzing the reaction with 0.02-0.2 *N* HCl. They mention that "one small fraction" of a partially methylated protein was still soluble and heat-coagulable. In the absence of controls and from the preceding quotation, plus the general knowledge of the effect of acid and alcohol on denaturable proteins, one can only surmise that most of their preparations were denatured in the process of methylation.

The reader may find of interest the following papers on methylation of proteins which do not come within the scope of this review (102, 76, 134-136).

*b. Carboxy Alkylation with  $\alpha$ -Halogen Fatty Acids.* Rapkine (138) found that the SH groups of proteins reacted with iodoacetate in neutral or slightly alkaline solution, just as Dickens (139) had reported for reduced glutathione.

The reaction may be indicated as follows:



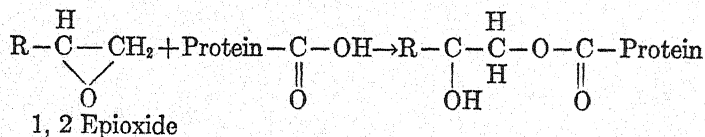
A number of investigators (46, 31, 140-142, 144, 352) have extended these studies to include such proteins as urease (46, 141, 32), crystalline lens protein (67), reduced keratins (141, 142), egg albumin (34) and tobacco mosaic virus (68). Michaelis and Schubert (140) showed that amino groups of amino acids are capable of reacting with mono halogen acetates at a measurable rate. Both hydrogens of the amino group can be replaced. Rosner (352) found, that after an initial rapid reaction with SH groups of denatured egg albumin, the iodoacetate reacted slowly with unknown groups.

Other  $\alpha$ -halogenated compounds have been used with equal success. These include iodoacetamide (140, 144, 141, 34, 68, 32), iodo ethyl alcohol (143),  $\alpha$  brom fatty acids, and benzyl and phenyl ethyl halides (142). All react with certain SH groups of proteins at pH 7.0-9.0 and physiological temperatures. The iodoacetamide is somewhat more reactive than iodoacetate (144). Both iodoacetate and the corresponding amide were found to react fairly rapidly with pyridine, even at pH 6.9 and 30°C. (145). Anson and Stanley (68) have reported that after almost complete inactivation of tobacco mosaic virus with iodoacetamide they could detect little, if any, drop in the number of SH groups. This calls for further study.

A great many enzymes (32, 46, 144-152) have been treated with iodoacetate or iodoacetamide. Depending on whether or not they were inactivated by this procedure, they have been classified as SH enzymes (151). Janssen (153) has recently reported that iodoacetamide inactivates the foot and mouth disease virus and that this inactive product served as a vaccine.

*c. Hydroxy Alkylation with 1, 2-Epioxides.* Fraenkel-Conrat (154) has found that 1, 2-epioxides such as ethylene and propylene oxides react with proteins in aqueous solutions at room temperatures and over a wide range of pH.

The reaction is, in general, as follows, though it is not known to which carbon of the epoxide the protein group is attached:



Some of these reagents are miscible with water and react with amino, tyrosine phenol, and SH groups, besides the carboxyl. Although the protein reacts with these reagents at any pH it is probable that different groups are reacting at different pH units. For example, Fraenkel-Conrat has indicated that  $\text{NH}_2$  groups react more readily in alkaline solution.

Reaction at room temperature between the protein and epioxide continues for several days, as is indicated by the slow drift in pH of the reaction mixture towards the alkaline side. The isoelectric point of the derivatives was 1-3 pH units more alkaline than that of the original protein.

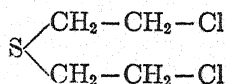
In Table I taken from the above work (154) is shown the way the various groups of crystalline egg albumin and crystalline  $\beta$ -lactoglobulin react with propylene oxide in neutral solution.

TABLE I  
1, 2-Epioxides  
Per Cent Decrease in Groups

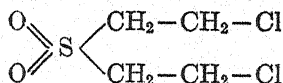
Groups	Egg Albumin		Lactoglobulin	
	1 Day	4 Days	1 Day	4 Days
COOH	44	70	49	66
NH <sub>2</sub>	63	73	88	72
OH (tyrosine) and indole	34	60	12	43
SH		100		100

It may be possible, by further studies, to choose conditions for making the action of this reagent more specific.

d. *Reaction with Mustard Gas and Mustard Sulfone.* Berenblum and Wormall (155) allowed mustard gas



and the corresponding sulfone



to act on serum at pH 8.0 and room temperature. The antigenic specificity of the treated sera differed from the original. There were no analyses made for the amount of either agent combined, nor did they attempt to determine what groups of the protein had reacted. They reasoned from the work of Cashmore and McCombie (156) and of Lawson and Reid (157), that the reaction was with the protein amino group.

More recently Herriott, Anson and Northrop (366) have found that chemically pure mustard reacts readily at room temperatures in aqueous solution with many different proteins. When the reaction mixture is kept at pH 5.5-6.0 the carboxyl groups are esterified, as indicated by

a decrease in the titration equivalent of the protein between pH 6.0 and pH 2.0. The number of mustard residues introduced was approximately equal to the decrease in number of carboxyl groups. These esters were rapidly hydrolyzed in solutions more alkaline than pH 9.0. Seven out of eight mustardized proteins showed no loss in Van Slyke amino nitrogen. In solutions of pH 7.6 Davis, Ross and Ball (367) found that in addition to carboxyl groups the histidine imidazole nitrogen of hemoglobin reacted with mustard.

Hellerman (368) has found that crystalline urease in dilute pH 7.0 phosphate buffer loses its "a" type SH groups (32) upon treatment with pure mustard. This change in SH groups measured with the aid of iodoso benzoate solutions did not result in the loss of enzyme activity.

Anson (369) found that, whereas crude or impure mustard reacted readily with denatured egg albumin SH groups, chemically pure mustard was much slower.

In a series of papers by Bacq, Fischer and Desreux, it was reported that mustard and in some cases other war gases, among which were mustard sulfone, the lachrimator chloracetophenone, and bromopicrin, reacted with the SH groups of such proteins as native egg albumin (370), denatured crystalline lens (371) and native urease (372, 373). When mustard acted on urease (373) a decrease in enzyme activity was noted which paralleled the reaction with SH groups. The difference between these results and those of Hellerman (368) is probably due to the higher concentration of mustard used by Fischer. Fischer (373) also states that mustard did not inactivate urease at pH 5.0-6.0. This is interesting in the light of the work reported above (366) in which the crystalline enzymes, pepsin, chymotrypsin, and yeast hexokinase were all inactivated by mustard at this pH. The interesting paper by Hartwell (396) concerning the action of mustard on proteins and amino acids was not seen in time to permit a discussion of it.

Kinsey and Grant (384) have noted that casein after treatment with mustard at pH 9.3 and subsequently refluxed with 1:1 HCl no longer supported growth of rats. They were able to show that the histidine, lysine, methionine and threonine were unavailable. When the treatment with mustard was performed at pH 7.4 the first three of the above amino acids were still unavailable but the threonine was free. Mustardization of horse serum globulin and albumin at pH 7.5 affected the methionine and lysine of these proteins.

Fixed rabies, hog cholera, and equine encephalomyelitis virus preparations inactivated by mustard at pH 7-8 were found by TenBroeck and Herriott (385) to serve as vaccines.

Publication of the interesting results of apparently induced mutations



brought about by sulfur mustard acting on drosophila (386), yeast (387) (388) and neurospora (389) and by the nitrogen mustard on bacteria (390) and neurospora (391) have just begun to appear.

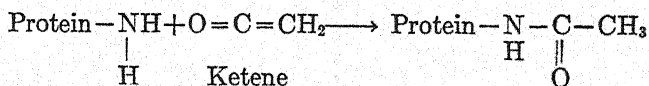
The reader is referred to review articles by Gilman and Phillips (392) and Dixon and Needham (395) for other results with these interesting reagents.

*e. Reaction with Dinitro Fluoro Benzene.* In an excellent paper, Sanger (374) has recently used for the first time the reagent, dinitro fluoro benzene which he finds reacts readily with proteins in aqueous bicarbonate at room temperature. From studies on amino acids he found that this reagent is capable of reacting with free amino, phenolic hydroxyl, SH and possibly imidazole groups. The great advantages of this reagent are that (a) the union with at least some groups (amino in particular) is stable to acid hydrolysis, and (b) the amino acid compounds are colored and may be readily separated by chromatography. It was reported that after treating insulin with this reagent and then subjecting it to acid hydrolysis followed by a quantitative separation of the derivatives, he was able to identify the dinitrophenyl derivatives of glycine, phenylalanine, and lysine. His figures showed that for an insulin subunit of 12,000 molecular weight there were two each glycine phenylalanine, and lysine residues. These results confirm those of Jensen and Evans (23) concerning the presence of free amino groups of phenylalanine in insulin and in addition demonstrates for the first time that glycine may be a terminal amino acid and thus contribute to the free amino nitrogen of a protein.

#### 4. Acylation

*a. Acetylation.* Acetylation of native proteins has been performed with ketene ( $\text{H}_2\text{C}=\text{C}=\text{O}$ ) and with acetic anhydride  $(\text{CH}_3\text{CO})_2\text{O}$ . The former reagent has been used extensively with aqueous protein solutions. Few, if any, cases have been reported in which acetyl halides have been used on native proteins.

*Ketene.* In solutions alkaline to pH 5, ketene reacts with the following protein groups arranged in order of decreasing reactivity:  $\text{NH}_2$ , SH, Tyr-OH. Its action on the amino group may be illustrated as follows:



The action of ketene on solutions of crystalline pepsin has been studied in some detail (4). It was shown that at pH 5.5 the amino groups of the protein were the first to be acetylated. The loss of the Van Slyke amino nitrogen was just equivalent to the increase in number of acetyl groups. This derivative was crystallized and it had the same enzyme



activity as the untreated pepsin. Some of its properties, such as solubility, were different. Further treatment with ketene under the same conditions brought about a decrease in peptic activity which could be reversed by treatment with strong acid at 0°C. During the reactivation the number of acetyl groups attached to the protein decreased and just equalled the number on the amino groups when the full enzyme activity was recovered.

In a later paper (5) it was found that acetylation of the tyrosine phenolic groups of pepsin was responsible for the loss of activity. This was determined through measurement of the tyrosine color value with Folin's phenol reagent at pH 8.0 where the phenolic esters of acetic acid are stable.<sup>4</sup> These esters are readily hydrolyzed at pH 11.0 and room

<sup>4</sup> A method was developed for the estimation of free phenol groups in acetylated pepsin and mixtures of tyrosine and diacetyl tyrosine. This method made use of Folin's phenol reagent, acting at pH 8. No color develops with diacetyl tyrosine, nor with other acetylated phenols. A few minutes at pH 11-12, however, hydrolyzes the acetyl groups, and the total phenol content can then be determined at pH 8.

Unfortunately, this method has been misinterpreted. In some instances after proteins have been treated with totally different reagents a mere change in the pH 8 color value has been presented as evidence of reactions between the reagent and the phenol group. It should be emphasized that a change in color value does not in itself mean that the number of phenol groups reacting has been altered. This has been amply demonstrated for pure tyrosine. Thus diiodotyrosine (7), N-carbo-benzoxy (93, 165), N-chloracetyl (93), N-benzoyl (165), phenyl carbamido (165), N-malonylated (93), and diazo (8) tyrosines have appreciably lower molar color values than pure tyrosine by the pH 8.0 methods, and yet the phenol group is essentially free in all these compounds. Just why these derivatives yield less color is a matter of conjecture, but steric hindrance as reported by Remington (166) may be an important factor.

Kleczkowski (167) has indicated that in iodinated serum globulin the diiodotyrosine yields no color with the Folin's phenol reagent and that the color obtained arises from the tryptophan. This is surprising in view of the fact that pure diiodotyrosine produces at least 50% of the color of tyrosine. It is possible that in this instance the tyrosine has been oxidized as well as iodinated. The phenol color value of tyrosine oxidized by iodine is reported (70) as 20% or less of the original tyrosine.

Native and denatured proteins are known to yield different quantities of color with the phenol reagent under identical conditions (168, 169). In these cases it has been assumed that the number of groups reacting with the reagent is different and that the color produced by any group is a constant. The lower value in native proteins could just as well be explained as resulting from a hydrogen bridge or similar structure depressing the reactivity of some or all the phenol groups.

Probably none of the above double interpretations are involved in the original experiment on acetylated pepsin for which the pH 8 method was designed. The reasons for this statement are:

- (1) Ketene is known to react with the phenol group of tyrosine (5, 170).

temperature. The close correlation between protein groups lost and acetyl groups introduced indicated that in two different derivatives no other groups were acetylated. When as many as twenty-three acetyl groups per mole pepsin were introduced, which reduced the peptic activity to less than 15% of the original, the number of amino plus tyrosine groups covered was less than the number of acetyl groups. It follows that some other protein group had reacted, but the nature of this group is not known.

It was also observed (4, 5) that the acetylation of the phenol group, and the inactivation, became slower with a lowering of pH of the medium and virtually stopped below pH 4.0. Due to the peculiar nature of pepsin, the pH could not be raised above 6.3 without encountering alkali denaturation.

The essentials of the work on pepsin have recently been confirmed by Hollander (164). He also showed that the enzyme specificity of pepsin on hemoglobin and synthetic substrates was not altered by acetylation.

Ketene reacts with the amino acid tryptophan resulting in a drop in its color value with Folin's reagent, but this reaction is not alkali reversible (5). Studies of ketene on other amino acids (170, 171), particularly those by Neuberger (171), reveal that aliphatic hydroxyl groups are apparently not readily acetylated but that SH groups are. Fraenkel-Conrat (172) has recently observed this reaction with the SH groups of native egg albumin. He further noted that it occurred at pH 3.3, as well as at pH 8.0, and that the linkage was alkali-labile. Contrary to the action of other acylating agents, the ketene reacted faster with the amino groups than with the SH groups. Li and Kalman (358) have just reported that phenolic groups in the lactogenic hormone are acetylated faster than the free amino groups.

All the amino groups of tobacco mosaic virus are not acetylated even after extensive treatment with ketene (Miller and Stanley, 92). Pappenheimer's (173) experiments on diphtheria toxin also indicate the presence of amino groups of differing reactivity. In the work on pepsin (4) it was concluded that all the amino groups were covered, for the final amino nitrogen value differed from the blank value by less than the experimental error.

There is some evidence (Miller, 169; Ross and Tracy, 174) that the acetylated phenol groups of proteins are hydrolyzed on long standing, even in nearly neutral solutions. Boor and Miller (175) have reported that

(2) The rate of liberation of phenolic groups by acid or alkali is the same for acetylated pepsin as for O-acetylated tyrosine (5).

(3) Both normal and acetylated pepsin are rapidly denatured at pH 8 (4).

freshly ketenized gonococcus still retained enough toxin to kill 2 out of 6 mice, but after standing a week in the cold, the preparation killed 6 out of 6 mice, indicating a reactivation of the toxin. Schramm and Müller (394) found that 1% active highly acetylated tobacco mosaic virus showed no recovery of activity when titrated to pH 9.-11., and placed at 35°C. for an hour. Since this treatment hydrolyzes acetylated phenols (5) they concluded that the inactivation of the virus was not due to acetylation of the tyrosine phenol groups.

Other native proteins and biological materials which have been treated with ketene<sup>5</sup> include: gonadotropic hormones (176, 177), insulin (178, 180), type I pneumococcus antibody (179), toxins of gonococcus and meningococcus (175), scarlet fever (62), shiga (180, 181), diphtheria (180, 182), tetanus (180), and ricin (180), antibrucella horse serum (180), and diphtheria antitoxin (183), parathyroid hormone (184), horse serum pseudo globulin (185) and tobacco mosaic virus (16, 92), foot and mouth virus (153), pancreatic amylase (186),  $\beta$ -amylase (187) phosphatase (188) and chymotrypsin (51).

*Acetic Anhydride.* Most of the literature concerning the use of acetic anhydride as an acetylating agent deals with insulin (190-193, 102-103) which is not inactivated readily by non-aqueous solvents. The protein has been suspended in glacial acetic acid, plus quinoline (103) or pyridine (190), after which the anhydride was added.

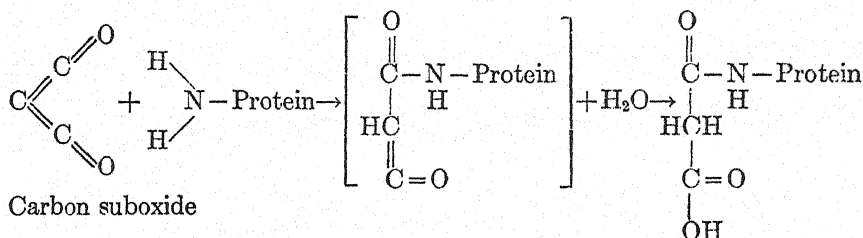
Hughes (377) has found that acetic anhydride was sufficiently soluble in water and hydrolyzed slowly enough to permit reaction with proteins, such as hemoglobin. This acetylation caused no denaturation, as indicated by its unchanged oxygen-combining capacity, but it did shift the isoelectric point to pH 5.0.

*b. Malonylation with Carbon Suboxide ( $C_3O_2$ ).* The preparation and certain chemical properties of this reagent have already been reviewed (195). It is similar to ketene in that it represents an internal anhydride and in that it reacts with the same type groups. It differs, however, in having two reactive foci, and there will therefore be a fraction of the reagent molecules which will react either with two groups of the same protein molecule or with two protein molecules. Experiments reported by Oncley, Ross and Tracy (196) supported the latter reaction. They found that after malonylating horse serum albumin, centrifugal analyses indicated the presence of about 12% of elongated dimers and 4% of either elongated trimers or symmetrical dimers. Previous work had shown that, in slightly alkaline solution, 75% of the total carbon suboxide combines with two moles of glycine (197). The above authors also

<sup>5</sup> Incidentally it has been demonstrated (189) that ketene is an extremely toxic gas, and adequate ventilation is recommended whenever it is used.

suggested that denaturation might explain these results. More recent studies (unpublished) indicate that denaturation is the more likely explanation, since acetic-anhydride-treated albumin showed similar ultra-centrifugal patterns.

The bulk of the reaction of  $C_3O_2$  with proteins may be illustrated as follows:



It may be seen that by this reaction free amino groups of proteins are converted to secondary amides and new carboxyl groups are introduced. Both these changes will tend to make the protein derivative more acidic in nature.

Ross and his collaborators have investigated the effects of this reagent on a number of proteins and amino acids (93, 174, 197-199). In their experiments with horse serum albumin malonylated at pH 7.5 (198), they found that the loss in amino nitrogen, determined by the Van Slyke method, was approximately equivalent to the malonyl groups acquired by the protein. They also presented titration curves of the untreated protein and of the derivative as evidence in support of the interpretation that the amino groups have been malonylated. It seems to this reviewer that the titration curves *do not* support this interpretation; for the number of groups titrated between pH 5.5 and pH 2.0, a measure of the total carboxyl value, is greater in the normal albumin than in the malonylated. Hence, there are fewer carboxyl groups in the derivative. In the region of pH 7.0-11.0, where free amino groups of protein may be expected to react, the malonylated protein shows more groups. The cause of these completely anomalous results is of some interest.

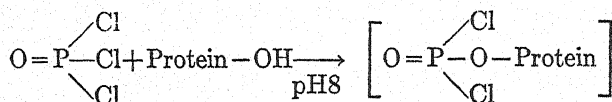
In this same paper (198) they also found that the amino nitrogen values of egg albumin and the amino nitrogen and activity of chymotrypsin decreased with malonylation at pH 7.5.

Tracy and Ross (93) have obtained better than 90% coverage of the amino groups of serum albumin and 60-88% coverage of the tyrosine phenol groups. The O-malonyl group on tyrosine in the protein appears to be easily hydrolyzed, since at pH 5.1 and 2°C., 50% was split in a month. At pH 8.2 and 37°C. all the malonyl groups were removed in two days. Pepsin (199) was similarly malonylated at pH 5.3. The

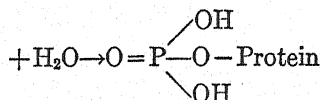
results were analogous to those obtained with ketene (4, 5). The O-malonyl tyrosine linkage in malonylated pepsin was hydrolyzed at a measurable rate and the peptic activity rose from 33% to 58% in 30 days.

Fraenkel-Conrat (200) has found that carbon suboxide reacts faster with SH groups of native egg albumin than with the free amino or tyrosine phenol groups.

*c. Phosphorylation with  $POCl_3$ .* This reaction takes place at pH 8.0-9.0 and 0°C.



Phosphorus oxychloride



Using a method similar to that reported much earlier by Neuberger *et al.* (201, 202), Rimington (203), dissolved the phosphorus oxychloride in 8 volumes of chloroform and added it to the protein over a 6 to 8 hour period with rapid stirring. After phosphorylation of caseinogen (casein), the protein contained 1% bound phosphorus. This derivative was readily dephosphorized by 1% NaOH at 37°C. for 24 hours. Phospho-nitrogen linkages are stable to alkali, and since there was no increase in amino nitrogen on dephosphorizing, Rimington concluded that amino nitrogens are not involved. The phosphorus of the derivative was easily liberated by the enzyme bone phosphatase—indicating an O-phosphorus linkage.

Using the above phosphorylating procedure, Heidelberger, Davis and Treffers (204) have phosphorylated egg albumin and found 20-30 phosphoryl groups bound per mole protein. The slope of the titration curve of the derivative was steeper than the untreated egg albumin in the region of pH 5.0-9.0. These investigators considered that there were insufficient hydroxy amino acids to account for all the bound phosphorus. Recent analyses (20) of egg albumin show 7.6% serine and about 4% threonine, or 31 serine and 14 threonine residues per mole protein. It is possible, therefore, that aliphatic hydroxy amino acid residues are the site of phosphorylation. Phosphoserine has been isolated from vitellin (205) and from casein (206), so that the phospho-aliphatic hydroxy linkage is not merely a product of the laboratory.

Mayer and Heidelberger (353) report the phosphorylation of crystalline serum albumin. They found that the use of potassium borate held the pH relatively constant during phosphorylation, but fewer phosphoryl

groups became attached than when the acid formed during the reaction was neutralized with sodium hydroxide. In their product of highest phosphorus content, containing 61 phosphorus atoms per mole protein, 28 were on primary amino groups, as shown by a decrease in amino nitrogen, determined by Van Slyke's nitrous acid method. The color value with Folin's phenol reagent also decreased during phosphorylation but the authors carefully noted that this is only an indication that the tyrosine phenol group is phosphorylated, and that other factors may be operating.

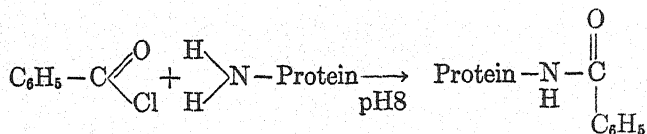
A very large proportion of all the phosphoryl groups became detached spontaneously from the protein. This spontaneous reaction was slowest at pH 8.0-9.0 and 0°C., and increased with temperature and acidity. At pH 7.4 and 37°C., 37 out of 48 groups from one preparation were split off in 13 days. In sharp contrast the remaining groups could *not* be hydrolyzed by 1% sodium hydroxide in 24 hours at 37°C., which procedure removes all the phosphorus from casein (354). One preparation after standing for some time had only 14 phosphorus atoms per mole protein, and all these were on amino groups. It was concluded that some of the labile, as well as the stable, phosphorus must either be attached to the protein amino groups or must shield them in some way to prevent their reaction with nitrous acid.

The titration curves of a phosphorylated serum albumin preparation showed an additional  $60 \times 10^{-5}$  equivalent acid groups per gram protein in the region of pH 2.0-2.5 and a similar increase between pH 5.0 and pH 8.0. Since there had been an increase of  $56 \times 10^{-5}$  equivalents of phosphorus per gram protein in this preparation, the authors point out that this increase of two titratable equivalents per phosphorus means that most of the phosphorus is linked to the protein as a mono ester.

If this interesting explanation for the difference in the titration curves is correct then the protein groups to which the phosphorus was attached must have been either non-ionizable, or must ionize beyond the range covered in the reported titration. Such would be the case if the phosphorus were bound to aliphatic hydroxyl groups, but if, as they suggest, the protein groups are amino and tyrosine phenolic, then a shift or change would be expected in certain regions of the titration curve *in addition* to the increases already observed.

*d. Benzoylation.* This reaction is known in the field of organic chemistry as the Schotten-Baumann reaction and is usually carried out at a slightly alkaline pH and low temperature.

Benzoylation has also been performed in pyridine solutions (136). It



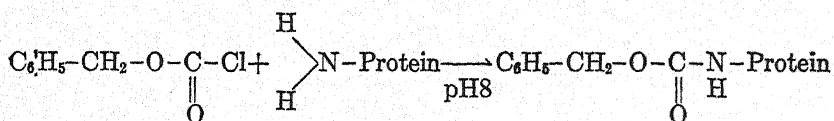
#### Benzoyl chloride

is claimed that protein derivatives prepared in this way contain more easily hydrolyzed benzoyl groups than when prepared in water. Blatherwick *et al.* (122) instead of stirring the benzoyl chloride into the solution shook the mixture until the benzoyl chloride disappeared, after which more was added. The shaking merely aids in dissolving the reagent, but this in turn speeds up the reaction with the protein. Goldschmidt and Kinsky (207) isolated  $\epsilon$ -N benzoyl lysine from benzoylated egg albumin.

Agatov (208) benzoylated tobacco mosaic virus and found no perceptible change in its activity. Miller and Stanley (209), using *p*-chlorobenzoyl chloride, obtained similar results. The latter's measurements of tyrosine phenol groups indicated that very few, if any, have been benzoylated.

Benzoylation inactivated the hormone gonadotrophin (210). See also papers by Lettré on the benzoylation of antigens (211).

*e. Carbobenzoylation.* The reagent used in this reaction is carbobenzoyl chloride.<sup>6</sup> Its reaction at pH 7.5-8.5 and at temperatures of 0-25°C. is illustrated below:



#### Carbobenzoyl chloride

This reagent, which was used so successfully by Bergmann and Zervas (212) in the synthesis of peptides, also combines with the amino groups of proteins (213). Gaunt and Wormall (214) studied the effects of this agent on insulin, egg albumin, and horse serum globulin. In the experiments on insulin they added several small quantities of reagent to a constant amount of insulin and noted the effect on the hormonal activity. The per cent inactivated increased linearly with the quantity of reagent added. Inactivation was nearly complete when twice the theoretical amount required for combination with the amino groups was added.

<sup>6</sup> A number of different chemical names have been applied to this reagent; all are correct but the use of so many is confusing. Some of these names are carbobenzoyl chloride (209, 215), benzyl chloroformate (214), benzyl carbonyl chloride (213), the acid chloride of benzyl carbonic acid (212), and carbobenzoyloxy chloride (216). Regardless of the merits of any particular name, the reviewer has chosen the one used most recently by workers in Bergmann's laboratory (215).

Miller and Stanley (209) studied the action of this agent on tobacco mosaic virus. The results appear to be similar to those obtained with other acylating agents.

One of the great advantages expected of the carbobenzoxy reagent is that after it is combined with the protein and its effects studied, it may be easily removed by non-hydrolytic means. Hydrogenation at pH 8-10 with the aid of a catalyst such as palladium black is the usual method. No report has been found of the removal of the carbobenzoxy group when attached directly to an amino group of a protein but its removal from O- $\beta$ -glucosido-N-carbobenzoxytyrosyl proteins has been accomplished (217, 218). In the hydrogenation reaction, CO<sub>2</sub> is liberated and quantitative measurements of the increase of the CO<sub>2</sub> have been used as a measure of the extent of the hydrogenation (217). A free amino group is also produced during hydrogenation.

More work on these reactions is clearly indicated. It is particularly important to be able to remove an attached group and to compare the regenerated protein with the original untreated material. If the examination shows them to be identical the chemical reactions are probably simple and not complicated by secondary or side reactions.

*f. Aryl Sulfonylation.*<sup>7</sup> Several compounds in this class have been studied. The conditions for reaction with proteins are similar to those used in benzylation. The reagents used include benzene sulfonyl chloride (219, 209)  $\beta$ -naphthalene sulfonyl chloride (220) and *p*-toluene sulfonyl chloride (221) and they may be indicated by the general formula R-SO<sub>2</sub>-Cl.

Gurin and Clarke (219) treated gelatin at pH 10.0 with benzene sulfonyl chloride. The amino nitrogen dropped nearly to zero and the sulfur content of the protein rose from 0.2 to 2.0%. There was no change in the Pauly test for histidine imidazole groups. The titration curves appear to be quite different, but this is an illusion. If one of the curves is shifted to coincide with the other in the region of pH 5.5 and all the other points corrected by a corresponding amount the two curves coincide except in the region of pH 8.5-11.5, where the  $\epsilon$ -N of lysine is supposed to react (18, 19). In this region the sulfonylated gelatin curve is lower by an amount just equivalent to the amino nitrogen lost by sulfonylation. In the present case any pH could have been used for comparing the curves except over the range pH 8.5-11.5. After acid hydrolysis of the sulfonylated gelatin, half of the lysine was isolated as the  $\epsilon$ -amino benzenesulfonyl derivative.

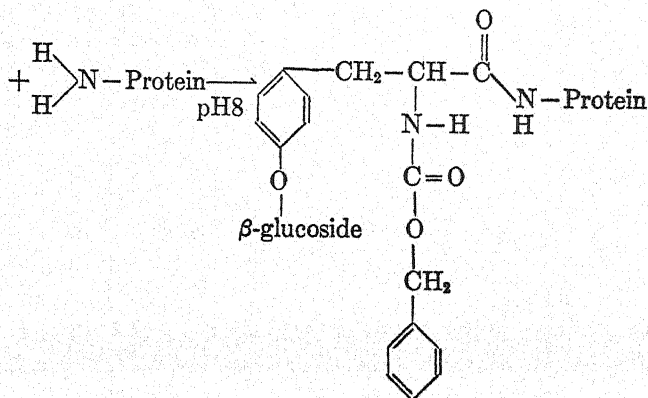
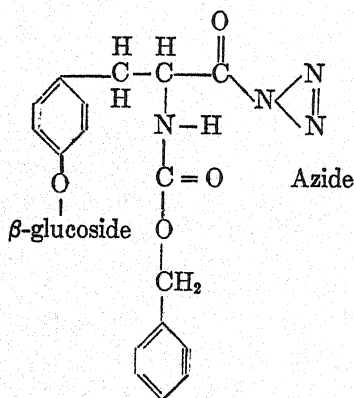
<sup>7</sup> The reader is also referred to the article by S. W. Fox in vol. 2, pp. 155-177, *Advances in Protein Chemistry*, in which he discusses certain reactions including aryl sulfonylation of terminal amino acids of peptides and proteins.



Christensen (221) has used *p*-toluene sulfonyl chloride on the polypeptide tyrocidine, and after hydrolysis has isolated oxy-*p*-toluene sulfonyltyrosine.

Miller and Stanley (209) after benzene sulfonylation of tobacco mosaic virus for various periods of time, found that the activity went down slowly but at two different rates, depending upon which of two hosts was employed for testing the derivatives. They also observed a 70% drop in the amino nitrogen and a 28% decrease in the tyrosine color value which did not return on treatment with dilute alkali.

*g. Acylation with Azides.* Clutton, Harington and Mead (216) started a series of interesting studies on the linkage of carbohydrates to proteins through an amino acid, such as tyrosine. The acyl-azide of *o*- $\beta$ -glucosidocarbobenzoxytyrosine dissolved in cold dioxane was stirred vigorously with a cold alcoholic protein solution at pH 8.0.



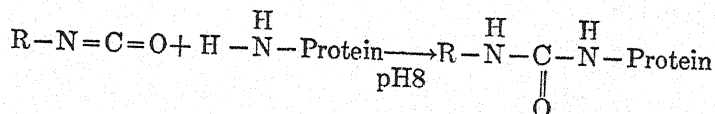
After completion of the reaction, the carbobenzoxy group was removed by reduction and the product contained 4.6% bound glucose. They believe that only  $\alpha$  amino groups will react at pH 8.0-9.0, for the  $\epsilon$ -amino groups of lysine are ionized at that pH. They indicate that of the 0.9% amino nitrogen (Van Slyke) only 0.3% is  $\alpha$ -NH<sub>2</sub>. This value for the  $\alpha$ -NH<sub>2</sub> groups is roughly equivalent to the number of tyrosyl glucosides added. In a second paper (217) serum globulin, albumin, and insulin were treated in cold aqueous solution with dioxane solutions of the azide. The insulin bound 11% glucose or 24 groups per mole insulin (40,000 molecular weight). This is quite interesting since Jensen and Evans (23) and Sanger (374) have found that some of the amino groups of insulin are  $\alpha$ -amino groups of phenylalanine and glycine. It is known that insulin contains about 20 phenylalanine residues, 27 glycine residues, and only 6 to 8 lysine residues, per mole protein (20, 382).

These gelatin and insulin derivatives were found to be antigenic and the insulin retained some activity.

Humphrey and Yuill (218) have similarly treated gelatin, insulin, and rabbit serum. Butler, Harington and Yuill (222) joined aspirin (acetyl salicylate) to rabbit and horse serum globulin by means of the azide reaction. 5% of the derivative was bound aspirin.

By means of the azide reaction N-carbobenzoxy 3,5 diiodothyronine was joined to horse serum globulin, albumin, and thyro-globulin (223). After iodination to form the thyroxyl compound anti sera to these, conjugated proteins were produced and the authors report that "passive immunization with anti sera to these derivatives protects against normal physiological effects of exogenously administered thyroglobulin and thyroxine."

*h. Reaction with Isocyanates.* Isocyanates react readily with amino groups of proteins in aqueous solution of pH 8.0 at 0°C. There are claims that they also react with protein SH groups (172) and with tyrosine OH groups (92). The reaction with amino groups may be illustrated as follows:



Hopkins and Wormall (224-226) in a series of studies have used phenyl isocyanate and *p*-brom phenyl isocyanate (224) on such proteins as caseinogen (casein) (224), horse serum albumin (225), and insulin (226). They found, in general, that good agreement was obtained between the loss in amino groups and the number of *p*-brom phenyl isocyanate residues introduced, as calculated from the bromine analyses. They also

obtained complete inhibition of the serological reaction between phenyl-isocyanate-treated horse serum albumin and its antiserum by use of the  $\epsilon$ -N-phenyl carbamido<sup>8</sup> derivative of lysine.

In studying the action of phenyl isocyanate on insulin, Hopkins and Wormald (226) observed that the amino nitrogen of the original insulin was several times greater than could be attributed to the lysine. Before they could isolate the unknown amino acid, Jensen and Evans (23) obtained the phenyl carbamido and  $\alpha$ -naphthyl carbamido derivatives of phenylalanine from crystalline insulin treated with phenyl and  $\alpha$ -naphthyl isocyanates. Jensen and Evans showed that, whereas there were 25 free amino groups per mole insulin before treatment with phenyl isocyanate, there were only 6 afterwards. The most recent amino acid analyses of insulin (20, 382) indicate that there are about 20 phenylalanine and only 6-8 lysine residues.

Sanger (374), using another reagent, has recently reported that the free amino nitrogen of insulin is distributed among lysine, glycine, and phenylalanine (see page 189 for a discussion of this work).

Roche, Michel and Schiller (375) have isolated the hydantoin of phenylalanine from phenyl-isocyanate-treated casein, globin, and ovalbumin. The amounts isolated per 100 g. of protein were 50, 15, and 100 mg., respectively.

Kleczkowski (227) has noted that not all of the amino groups of some proteins react with phenyl isocyanate. With horse serum the Van Slyke amino nitrogen value decreased from 5.7% of the total nitrogen to 2.3%, and with crystalline egg albumin from 6.0% to 1.8. Since similar results were obtained with formaldehyde, these findings suggest two types of amino groups with different chemical properties.

Schramm and Müller (16) exposed tobacco mosaic virus to phenyl isocyanate at pH 7.5-8.0 until the ninhydrin and nitrous acid amino nitrogen tests were negative. The virus was still active. In extending these studies, Miller and Stanley (92) have noted that the tryptophan of the virus was not affected but that the tyrosine phenol value decreased.

Fraenkel-Conrat (172) has reported that phenyl isocyanate and certain acylating agents react with SH groups of native egg albumin even faster than with amino groups. This reaction with SH groups appears to be only slightly affected by pH since the reaction was observed at pH 3.3 as well as at pH 8.0 (228). These thiol derivatives were found to be alkali-labile. No change was observed (228) in the acidic groups of egg albumin nor in the tyrosine phenol groups, as had been noted by Miller and Stanley (92) for tobacco mosaic virus treated with phenyl isocyanate.

<sup>8</sup> Phenyl carbamido, phenyl carbamino, and phenyl ureido are names for the same phenyl isocyanate derivative of amino groups.

The failure to observe a drop in tyrosine color in egg albumin is of interest for, as is discussed in a footnote on page 179, the phenolic groups of this protein appear to react only with certain reagents. The treatment of dried proteins with phenyl isocyanate in pyridine at 70°C. is not discussed here (228).

Creech, Franks and their collaborators have prepared isocyanates of a large number of known carcinogens related to anthracene and 1, 2, 5, 6-dibenzanthracene (10-13, 229, 230). These isocyanates have then been coupled with such proteins as casein (230), horse (12) and bovine serum albumin (229), egg albumin (229), and anti type III pneumococcus rabbit serum (229); and antisera to them have been prepared. Coupling at pH 8.0 was aided by the addition of dioxane.

They were able to introduce (12) twelve to fifteen 1, 2-benzanthryl-10-carbamido groups into horse serum albumin at pH 8.0 and nearly 30 at pH 10.0, while only 4 or 5 of 3, 4-benzpyrenyl-5-carbamido groups went into the same protein at pH 8.0.

Very few animal protection experiments have been reported; for this one can only surmise that the war is responsible. In an early paper (230) they reported that mice immunized with a casein-1, 2; 5, 6-dibenzanthracene antigen were less susceptible to the carcinogenic action of the free hydrocarbon.

The readers may find of interest the work of Vellez using phenyl isocyanate on diphtheria and tetanus toxins (231), and that of Gutman (232), in which clupein containing no lysine reacts with phenyl isocyanate. Mutsaers and Gregoire (233) have employed  $\alpha$ -naphthyl isocyanate. Sizer (51) has found that, whereas a two to one concentration ratio of chymotrypsin to phenyl isocyanate did not result in inactivation at pH 7.6, 0°C., a two to ten ratio did. Pancreatic amylase (234) and "alkaline" phosphatase (188) have also been treated with phenyl isocyanate.

### 5. Reaction with Aldehydes<sup>9</sup>

Considering the number of papers on the subject and the importance of this type of agent in the formation of toxoids and vaccines, etc., it is surprising to find how little is really known about the reactions of aldehydes with proteins. The explanation is probably twofold. First, aldehydes can act in several different capacities. They may bind  $\text{NH}_2$ ,  $\text{NH}$ , and  $\text{SH}$  groups and act as reducing agents, and occasionally as oxidizing agents. Secondly, most of the studies have been made with formalde-

<sup>9</sup> The reader is referred to the much more complete discussion of the reaction of formaldehyde with amino acids and proteins by D. French, and J. T. Edsall in *Advances in Protein Chemistry*, vol. 2, pp. 277-335. The present manuscript was completed before this article was seen.

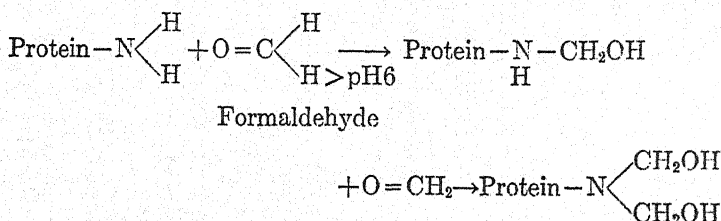
hyde. Being small in size, having no identifying property once it is bound, and being difficult to remove it has not been possible to follow adequately the quantity of formaldehyde introduced into the protein. As soon as radioactive carbon becomes more plentiful it should be possible to circumvent the second of these obstacles, and really establish the nature of the groups in proteins that react with formaldehyde and to determine the structural difference between the reversible and irreversible complexes.

A general summary of the nature of the reaction of aldehydes with proteins is discussed below.

In aqueous solutions more alkaline than pH 6,<sup>10</sup> aldehydes react with the amino groups of proteins. In many instances not all amino groups react (227, 173, 26, 236).

The titration curve of formolized egg albumin deviates from that of normal protein in the region of pH 6-10, and Kekwick and Cannan (237) have indicated that of all the titratable groups only the amino groups combine.

Although it has generally been assumed that the reaction with amino groups results in a methylene ( $-N=CH_2$ ) linkage, Levy's kinetic studies (238, 239) indicate that two formaldehydes may combine with each amino group. A methylene type union would appear to preclude the addition of a second aldehyde, but with a methylol (240) structure ( $-NH-CH_2OH$ ) no such impasse exists.



Triformals have been reported (241) and isolated in the case of amino acids (242, 243). It may be that the methylol structure is the reversible form obtained soon after the aldehyde and protein are mixed and that this changes with time to an irreversible methylene structure. No good evidence on this point has come to our attention.

The number of amino groups combining increases with the aldehyde concentration up to about 1.25 molar (237) in one instance, and 3.0 molar in another (244).

<sup>10</sup> This is the equilibrium pH after the formaldehyde is added to the amino compound. It has been claimed that amino acids do not react below pH 7 (235). In this case the pH was probably determined before adding the formalin for afterwards the acidity may drop to pH 6

Besides the amino groups, a number of other protein groups have been reported as reacting with aldehydes. Although there is frequent reference to the original reports there has been little or no attempt to confirm these findings. SH groups of denatured egg albumin are reported (245) as reacting with formaldehyde, but no case has been established for native protein. However, the reaction of aldehydes with simple SH compounds such as cysteine is well established (246-249). The histidine imidazole NH group of proteins has likewise been reported as binding aldehydes (250). If this is so the titration curve of the histidine-rich protein hemoglobin should change markedly in the region of pH 5.5-7.5 on the addition of formaldehyde. Kekwick and Cannan (237) did not specifically mention the imidazole group in their titrimetric study of formalized egg albumin, but they did find 1-3 groups<sup>11</sup> which reacted with formaldehyde and which titrated at a more acid pH than most of the amino groups. From the most recent analyses (20) egg albumin contains five histidine residues per mole protein. Similar results appear in the titrimetric study of  $\beta$ -lactoglobulin (244).

Reaction of aldehydes with the indole NH group of tryptophan in proteins has been reported in tetanus toxin (251) and tobacco mosaic virus (26). In the latter instance, the Folin's phenol reagent color value decreased as the reaction with formaldehyde proceeded, and increased with removal of the aldehyde by dialysis in dilute acid. Indole, pure tryptophan, and glycyl tryptophan behaved in a similar manner, but not tyrosine nor glycylytyrosine. Benzaldehyde, acetaldehyde, propionaldehyde, and butyraldehyde were also used in this work with similar effect. Reversal of formaldehyde inactivation of tobacco mosaic virus could not be confirmed (356). It was also claimed that the drop in phenol color could not be correlated with a decrease in virus infectivity.

In addition to Levy's studies referred to above (238, 239), the kinetics of detoxification of staphylococcus toxin by formaldehyde have been studied in some detail (252). The results show that the rate of reaction is directly proportional to the concentration of formaldehyde and to the square root of the hydroxyl ion. The reaction has an unusually high temperature coefficient of 4-5 for a change of 10°C. It is stated that the reaction follows a unimolecular course. This is true only when either the aldehydes or proteins are present in great excess, for the reaction should be bimolecular. Similar results have been obtained with diphtheria

<sup>11</sup>These workers reported 1-2  $\alpha$ -amino groups and 16-18 lysine  $\epsilon$ -amino groups per mole egg albumin. Since that time the molecular weight has been assigned a value of 43,000. (18). The number of groups should therefore be multiplied by the factor 43,000/34,500 or 1.25.

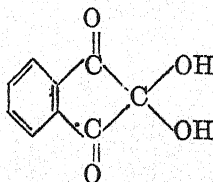
toxin (253), but in this case it is reported that the reaction is catalyzed by both hydroxyl ion and formaldehyde.

Increasing the molecular weight of the aldehyde is reported as depressing the rate of detoxification and the firmness of union with diphtheria toxin (254).

Hewitt (255) showed that diphtheria toxin was inactivated by formaldehyde but not by sodium hydrosulfite or  $H_2O_2$ . The oxidizing or reducing powers of formaldehyde were therefore presumably not responsible for its action. It was also claimed (236) that toxoids formed at pH 6.3 bound more antitoxin than those prepared at pH 8.6.

The reader may find of interest some of the following papers in which aldehydes (principally formaldehyde) have been allowed to act on serum proteins (256); gelatin (257, 246); the enzymes pancreatic amylase (186) and chymotrypsin (51); antibodies (179, 258); the hormones, insulin (259, 102, 260), gonadotrophin (116), lactogenic (261), and luteinizing (262); the toxins of purified diphtheria (173, 236, 253, 255), tetanus (251), and meningococcus (263) and the viruses of eastern equine encephalomyelitis (264), potato X virus (265), influenza (266, 267), foot and mouth disease (153), vaccinia (268), *Jap. B. encephalitis* (269, 270, 379), typhus (357), and scrub typhus (380).

Ninhydrin, triketo hydrindene hydrate, has many of the properties of aldehydes,



and is most often employed to determine qualitatively the presence of  $\alpha$  amino groups with which it unites to form a colored compound.

Duliere (271) reported that human serum proteins treated with ninhydrin in slightly alkaline solution for 2 days at  $37^\circ C$ . no longer reacted with formaldehyde and *vice versa*. Eggerth (272), in a fairly complete study, found that optimal conditions were  $37^\circ C$ ., pH 7.0-7.5, 1% ninhydrin, and two days or more for reaction. Borate buffers should be avoided.

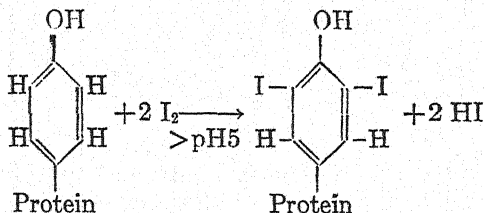
The ninhydrin serum protein derivatives are insoluble at pH 4.5-5.0. All protein amino groups are not covered by ninhydrin. Eggerth agrees with Ross and Stanley (26) that formaldehyde-treated proteins still react with ninhydrin.

### 6. Halogenation

*Iodine* has been used much more than any of the other halogens and will therefore be discussed in much greater detail.

The action of free halogens on proteins in aqueous solution can theoretically take any of three paths: (a) addition, (b) substitution, or (c) oxidation. The reviewer does not know of a case involving addition, but as more proteins with peculiar structures are studied cases may arise. To detect the simple addition of halogen to proteins one might expect a loss of free halogen from solution without a simultaneous formation of iodide ion. In substitution, the iodide ion formed just equals that which is organically bound to the protein and is just half of the decrease in free iodine. With oxidation there is no organically bound iodine, and the iodide ion formed equals the loss of free iodine. Undoubtedly, both oxidation and substitution take place under some circumstances and with certain proteins. In such cases precise estimates of the loss in free iodine, organically bound iodine and iodide ion formed should permit a calculation of the extent of each reaction. Such a study was made on pepsin (6).

Although earlier workers iodinated proteins, Ostwald in 1910 provided the first unequivocal proof that tyrosine in proteins was iodinated. He isolated diiodotyrosine from iodinated albumin (273), gliadin (274), and casein (275). Since then papers on serum albumin, egg albumin, and serum globulin (276), pepsin (6, 7), insulin (125), and the lactogenic hormone (277) all indicate that the action of iodine on these proteins in neutral or slightly alkaline solution is one of substitution on the tyrosine residues. The reaction is as follows:



The Millon's test (27) is negative when all the tyrosine in a protein is iodinated (278-280). This serves as a convenient method of detecting completion of the reaction.

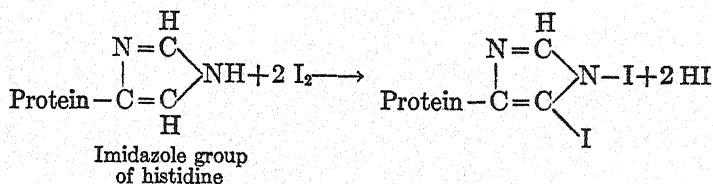
In addition to the action on tyrosine, there is evidence that histidine in some proteins reacts with iodine under certain circumstances. Thus, Pauly (281) found that imidazole and the imidazole nitrogen of benzoyl histidine react with iodine. In the latter compound two iodines substituted; one for the hydrogen on the imidazole nitrogen and the other on



a ring carbon. The iodine on the imidazole nitrogen was easily removed by bisulfite.

In proteins, Blum and Strauss (282) found that iodination in bicarbonate buffer for 24 hours at 40-45°C. yielded products which even after purification and washing contained relatively high iodine values. These values dropped on treatment with bisulfite to that obtained by iodinating for only six to nine minutes. Bauer and Strauss (283, 284) then studied the iodination of globin (from hemoglobin) which is rich in histidine. They found that it absorbed much more iodine than expected from the tyrosine analyses. They assumed that the histidine absorbs two iodines. Oddly enough, they found the iodine analyses of iodinated serum albumin, serum globulin, and egg albumin agreed with that expected from the tyrosine content of these proteins, yet they all contain histidine. Iodinated insulin showed no evidence of containing iodohistidine (125). Recent reports of the histidine content of the hormone place the value at about 4% (20). The histidine content of pepsin is very low—probably only one or two residues per mole protein (285), and iodination of so small a quantity would be difficult to detect.

The reaction with histidine is supposed to be as follows:



The suggestion has also been made (283) that the  $\alpha$ -CH of tryptophan in proteins is oxidized, for the Adamkiewics and Ehrlich tests are negative after iodination. If this is so, chymotrypsinogen which is rich in tryptophan should be interesting to study.

In addition to all the other groups noted by them, Blum and Strauss (282) also reported a disappearance of "unoxidized sulfur," and stated that the iodide ion formed during iodination was over four times that expected from a straight substitution reaction. They suggested that cystine sulfur had been oxidized. They also found that the biuret value was lower in the iodinated protein. Sometimes considerably more iodine is bound to proteins than can be accounted for on the basis of their tyrosine and histidine content (286, 287).

Since the nature of the reaction of iodine with proteins is affected by variations in the medium, a discussion of these variables is appropriate.

Iodine may be added to protein solutions in a variety of forms. Pulverized iodine or a concentrated solution in alcohol introduces free iodine

with a minimum of iodide ion. Iodine in KI or  $I_3^-$ , as it is sometimes designated, hypoiodite, and iodine in KI plus strong ammonium hydroxide, which may involve an intermediate  $NI_3$ , are other forms. There have been no first rate comparisons of the iodinating effect of these various forms under otherwise comparable conditions.

It is well known that the oxidation potential of iodine, as with most oxidants, decreases as the pH increases. On the other hand, the rate of substitution of iodine increases with increased pH. Thus, if only oxidation is desired, the medium should be as acid as possible without introducing secondary complications, while, if oxidation is to be avoided and only substitution is desired, the pH should be as alkaline as possible.

In keeping with the above generalization, Anson (34) has oxidized the SH groups of proteins with iodine at pH 3.2 with no detectable substitution. Harington and collaborators (223) have recommended strong ammoniacal solutions for substitution with a minimum of oxidation. Li (288) has reported that the rate of substitution into tyrosine actually decreased in dilute alkali. Brand and Sandberg (289) obtained erratic results in alkaline solution, but at pH 6.8 iodination of insulin was reproducible.

In many instances, proteins cannot withstand extremes of pH without becoming denatured and must be treated in more nearly neutral solutions. Swine pepsin, an extreme example, is rapidly denatured in solutions more alkaline than pH 6. However, it was found (6) that substitution without oxidation took place rapidly above pH 5. Below pH 4.5 there was practically no substitution. In one experiment (unpublished) with pepsin of constant solubility, substitution was obtained at pH 5.5 with *not more* than one group per mole protein being oxidized. This should not be taken as evidence that one group of pepsin is oxidized, for the values were too small. Instead it is positive evidence that practically no oxidation occurred with pepsin under these conditions. Shahrokh (290), however, observed considerable oxidation during iodination of crystalline serum albumin at pH 6.5. He also noted that increased temperature favored oxidation. The high temperature coefficient of 10 for a change of  $10^\circ\text{C}$ . for the oxidation of tyrosine by iodine (70) tends to confirm this. In both the above experiments oxidation was determined by the appearance of more iodide ion than expected from the loss in free iodine and protein-bound iodine on a basis of straight substitution. Alcoholic iodine containing no iodide ion was used in the experiments on pepsin. This made the estimation of iodide ion formed during the reaction more precise.

Clutton, Harington and Yuill (223) in favoring the use of ammoniacal solutions of iodine for iodination have suggested that in "dilute alkaline solutions" oxidation by hypoiodite is encountered. This is surprising

since Cofmann (291) and recently Li (288) have reported hypoiodite as being an extremely effective iodinating agent for phenols. Even the iodinating action of  $NI_3$  (which is momentarily formed by the addition of strong iodine in KI to ammoniacal solution) is attributed to the hypoiodite which results from its action with water (291, 292).

Interesting results were obtained by Ludwig and Mutzenbecher (293) who isolated thyroxine and mono iodotyrosine from iodinated casein. This isolation of thyroxine has been confirmed (294) and although the mechanism of its formation is still uncertain most workers are agreed that iodination of tyrosine is one of the first steps (295, 296).

Crystalline mono iodotyrosine was also isolated from pepsin treated with a small quantity of iodine (7). Some of its properties did not agree with those of synthetic mono iodotyrosine so that its exact nature has been questioned (297). The isolation from pepsin has been repeated and a comparison made with a synthetic product. Such properties as solubility, ultraviolet absorption spectra,  $pK$ 's, and distribution coefficients were compared and cross-examined wherever possible. There can be no doubt that the crystalline product from pepsin is mono iodotyrosine (298).

Iodination of proteins has produced measurable changes in the titration curves. In zein (280), insulin (125), and pepsin (6) the region of the titration curves usually assigned to the phenolic group of tyrosine ( $pK=10$ ) is displaced in iodinated proteins in the direction of increased acidity by nearly 2 pH units. This is apparently not true for iodinated globin (299).

The phenol groups of globin may be muzzled in a manner similar to that noted for some other proteins such as egg albumin (see next paragraph). In view of the results on globin, it would be of interest to determine if the titration curve of egg albumin is markedly altered by iodination. Only a few phenol groups of egg albumin are detectable by titrimetric (74), or spectrophotometric (75), methods so that unless iodination brings about the release of all the phenol groups, only a slight shift corresponding to the few phenol groups would be expected. Fortunately, egg albumin is not denatured at an appreciable rate below pH 12.0 (75), so that the experiment would not be complicated by that reaction.

Rate measurements on pepsin and serum albumin have led Li (300) to conclude that all tyrosines in a given protein molecule are not iodinated at the same rate. He also suggests that "availability" of groups in the protein may be a factor. The "availability" or "reactivity" of protein groups is receiving considerable attention. Anson (34) and Greenstein and Edsall (83) have discussed it in connection with SH determinations while Crammer and Neuberger (75), Cannan (74), Philpot and Small

(8) and Li (300) have some interesting evidence for variability of tyrosine phenol groups of proteins.

Harington and Neuberger (125) were able to reduce some of the iodine from iodinated, practically inactive insulin, and thereby to reactivate the hormone partially.

From completely iodinated pepsin after alkaline hydrolysis and partial purification at least 82% of the total iodine was accounted for in a solution analyzing as diiodotyrosine (7). 53% was actually crystallized.

Iodinated serum albumin (301, 290) and iodinated pepsin (7) have been crystallized and some of their properties examined. In the latter instance the electrophoresis and solubility tests indicated a high degree of homogeneity; yet it was clearly different from the original pepsin.

A number of other papers which the reader may find of interest deal with the action of iodine on insulin (302, 131), chorionic gonadotrophin (303), scarlet fever toxin (62), serum proteins (167) and tobacco mosaic virus (77, 68).

Investigations in which *bromination* was used are confined mainly to serological studies after bromination of serum proteins (304, 279, 305, 306).

It must be evident to the reader that more work is needed to determine the amino acids *other than* tyrosine in proteins which react with iodine. Since iodine has a powerful effect on some absorbing groups, spectrophotometric analyses coupled with variations of pH and other factors might prove very useful. In the case of tyrosine, the ionization constants, or pKs of phenolic groups of the iodo derivatives have been determined spectrophotometrically (75, 307). For tyrosine (75, 308), mono iodo-tyrosine (308), and diiodotyrosine (75, 308) the pKs are 10.1, 8.2 and 6.4 respectively.

## 7. Nitration

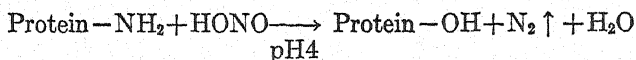
Although there are numerous references to the preparation of nitrated proteins, they have in nearly every instance involved the use of strong nitric acid which surely brought on drastic internal changes, besides nitration of the tyrosine residues. Wormall (279) in 1930 used tetranitromethane to nitrate serum proteins. He merely shook into an aqueous pyridine solution of serum an excess of tetranitromethane at room temperature and kept the solution slightly alkaline by the addition of pyridine, as needed. This reagent appears to have been forgotten by protein chemists. In the absence of experimental evidence, it is presumed that the nitro groups were introduced into the benzenoid ring of tyrosine in positions ortho to the phenol group. Recently it was reported (393)

that tetranitromethane reacted reversibly with diphtheria toxin but failed to react with amylase.

### 8. Deamination

It had generally been supposed from the classical work of Van Slyke (22) that the free amino groups of proteins were the  $\epsilon$ -amino groups of lysine. Wiley and Lewis (309) found that lysine could not be detected in the hydrolyzate of deaminized casein. However, the work of Jensen and Evans (23) and Sanger (374) demonstrates that in insulin many of the  $\text{NH}_2$  groups are  $\alpha$ -amino groups of phenylalanine and glycine. In this connection see also Chibnall (381) and Brand (382).

The deamination with *nitrous acid* can be performed on those proteins capable of remaining native at pH 4.0-5.0. The reaction is illustrated below.



Philpot and Small (8) in a study of the action of nitrous acid on pepsin at pH 4.0 have separated the deamination from subsequent reactions. Their procedure is, therefore, to be preferred since very few investigators have appreciated the existence of more than the one reaction. They found that at pH 4.0,  $N/1$   $\text{NaNO}_2$  at  $0^\circ\text{C}$ ., or  $N/16$   $\text{NaNO}_2$  at  $38^\circ\text{C}$ ., completely deaminized pepsin in a half hour.

Others (51, 62, 122, 186) have used related procedures. In one instance (131) isoamyl nitrite was used in place of the  $\text{NaNO}_2$ .

Deamination replaces a titratable polar amino group by a non-polar relatively inert hydroxyl group. This change is shown in the titration curves (257, 310, 355).

The reaction of nitrous acid with amino groups is a trimolecular reaction which reduces to a pseudobimolecular reaction in the presence of an excess of nitrous acid (311).

The diazotization reaction (discussed in the next section) is bimolecular, but becomes unimolecular in the presence of excess nitrite (8). Little and Caldwell (234) made use of this difference in order of reactions to decide that the inactivation of pancreatic amylase by nitrous acid is primarily a deamination reaction. Sizer (51) found the inactivation of chymotrypsin to be a first order reaction, indicating that the diazotization reaction was responsible for the loss of activity.

Weil and Caldwell (187) observed that the rapid inactivation of  $\beta$ -amylase by nitrous acid could be completely reversed by  $\text{H}_2\text{S}$ , showing that the oxidizing action of this reagent is also a factor to be considered.

Other papers of interest in this connection are on insulin (259, 122, 76),

tobacco mosaic virus (77, 394), scarlet fever toxin (62) and lactogenic hormone (312).

### 9. Direct Diazotization

It has long been postulated that nitrous acid forms nitroso or diazo compounds with proteins (313-315, 8), in addition to deaminizing. Tyrosine (316, 317) and tryptophan (318, 8) are known to form diazo complexes when treated with  $\text{HNO}_2$ . Philpot and Small (8) have shown that after deamination nitrous acid forms diazo structures in positions ortho to the phenolic groups of tyrosine in pepsin. Using  $N/16$   $\text{NaNO}_2$  at  $38^\circ\text{C}$ . or  $N/1$  at  $0^\circ\text{C}$ ., these investigators treated solutions of crystalline pepsin at pH 3.8-4.6. The peptic activity did not change during deamination, but on further treatment it dropped gradually to 50% of its original value and remained there. Fortunately or otherwise only half of the total tyrosine residues were found to be diazotized. The authors suggest that the other half are not free to react in the protein. Li (300) has come to a similar conclusion from iodination experiments. Diazo pepsin is yellow, as are most proteins after treatment with  $\text{HNO}_2$ , and has a new absorption at  $411.5\text{ m}\mu$  (8). The resulting product is a true diazo compound, as shown by its coupling with certain phenols in alkaline solution, and giving a red color upon being irradiated in alkaline solution. No color develops if it is irradiated in acid solution. The authors claim this test to be characteristic of *o*-diazo-*p*-cresol and *o*-diazo deamino tyrosine. Philpot and Small studied the kinetics of the reaction from many angles and put great stress on the quantitative similarity of the reaction of nitrous acid on pure tyrosine and on pepsin. They visualize the reaction as taking place in two steps—first a nitroso group is formed and then nitric oxide, which is always present in solutions of nitrous acid, changes the nitroso group rapidly to the diazo compound. These workers reported that reduction of the protein diazo compound with  $\text{Na}_2\text{S}_2\text{O}_4$  to the corresponding hydroxy compound did not alter the enzymatic activity of the product.

Morel and Sisley (316, 319) suggested that after the nitroso compound is formed it is reduced to an  $\text{NH}_2$  group and then diazotized to the diazo compound. They also found that reduction of a diazonium salt of tyrosine with sodium hydrosulfite resulted in the compound 3-amino tyrosine, instead of the corresponding hydroxy compound indicated by Philpot and Small (8).

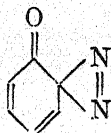
Concerning just what groups of proteins are diazotized, there appears to be some difference of opinion. Wiley and Lewis (320) state that casein treated with nitrous acid had lost some of its histidine, as determined by four different methods. Some of the tyrosine in the casein also appeared to have been affected. Eagle (318) suggested that tryptophan is an im-

portant reactant, and points out that after reaction with  $\text{HNO}_2$  tryptophan couples readily with  $\alpha$ -naphthol. He states further that tryptophan reacts under conditions which bring about similar changes in the proteins, egg and serum albumin and casein, but not in tyrosine, or in zein which contains tyrosine but not tryptophan. Eagle believes a nitrosamine is formed with the  $\text{NH}$  group of tryptophan. This evidence of Eagle's is interesting since Wiley and Lewis (320) found no change in the tryptophan values, and Steudel and Schumann (321) observed no change in either tyrosine or tryptophan.

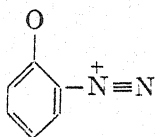
Philpot and Small (8) noted that tryptophan reacts nearly 50 times as fast with nitrous acid as does tyrosine and that it also becomes yellow but concluded from rate measurements that it was not important in the inactivation of pepsin.

The results of Philpot and Small (8) are strong evidence that tyrosine can be diazotized. However, it would appear that careful studies are called for to determine just what other groupings may react. Spectrophotometric examination of a number of treated proteins whose amino acid composition is known should prove interesting. In this connection, it should be recalled that Philpot and Small found that in diazotized pepsin there was an absorption at  $411.5 \text{ m}\mu$ , attributable to the diazo structure. This fact has been used by Li *et al.* (312), in their studies of nitrous acid on hormones and by Little and Caldwell (186), who compared the rates of inactivation of pancreatic amylase by nitrous acid and of diazotization of tyrosine.

The reviewer (322) has diazotized both tyrosine and 3-amino tyrosine (323) by the method of Philpot and Small (8). The amino tyrosine reacted much more rapidly as might be expected, and the absorption curves were similar, but not identical. Diazotized tyrosine showed a peak at  $390$ , whereas the corresponding peak in diazotized 3-amino tyrosine was at  $400 \text{ m}\mu$ . The absorption curves of these products coupled to resorcinol showed the same peak at  $500 \text{ m}\mu$ . Although equivalent quantities of the two materials were used, the absorption density of the 3-amino product was higher in the region of  $400 \text{ m}\mu$  where the diazo group absorbs (8). This probably means that diazotization of tyrosine was not complete. Taylor and Baker (324) indicate that diazo phenols are a special class of diazo compounds. They state that it is pretty generally agreed that these compounds resemble aliphatic, rather than aromatic, diazo compounds in that quinone structure



or possibly the betaine type structure,



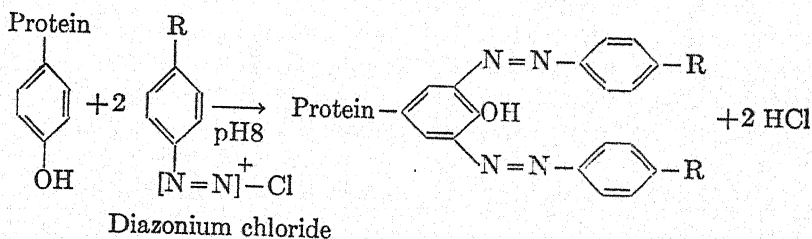
accounts for their properties.

In our experiments (322) diazo tyrosine or diazo pepsin have not coupled readily, if at all, with tyrosine or proteins containing tyrosine. It had been previously noted (8) that diazo tyrosine does not couple with certain monohydric phenols.

#### 10. Coupling with Diazonium Salts

Probably no chemical reaction of proteins has been used more than he coupling with diazonium salts at a neutral or slightly alkaline pH and at low temperature. The ease with which chemical structures of simple or complex nature can be joined to proteins is largely responsible for its wide use. Landsteiner (1) and other immunologists have made literally hundreds if not thousands of these azo-protein compounds. The literature is so vast that merely the nature of the reactions and the effect of the variables will be discussed here. For more details the reader is referred to the modern text books of Landsteiner (1) and Boyd (325).

The general nature of the reaction with tyrosine in proteins is illustrated below:



As shown, coupling takes place at the positions ortho to the phenol group of tyrosine. Pauly (326-328) and Inouye (329) indicated that besides coupling with the benzenoid system of tyrosine two moles of diazonium salts can couple with histidyl residues in proteins.

Boyd and Hooker (330) and later Boyd and Mover (331) observed that after exposure to either an excess of diazonium salt or to repeated treatment, more diazonium had coupled to the protein than could be accounted for from the tyrosine and histidine analyses. Eagle and Vickers



(332) were able to show that amino groups of nearly any amino acid bind diazonium salts. Their results indicate that two diazonium groups combine with each amino group. Busch *et al.* (333) isolated the compound with glycine. It had two azo groups to each glycine. In addition to the amino groups, Eagle and Vickers found that the NH group of indole, tryptophan, proline, and hydroxy proline, reacts with diazonium salts. All of the resulting compounds except for tryptophan were colorless, or at least no more colored than the diazonium salt.

Thus far the work has shown that protein groups might couple with diazonium salts. Further studies should produce methods for identifying the protein groups that *do* couple. Again spectrophotometric studies might prove valuable.

In general the pH of the coupling medium should be slightly alkaline. Coupling has been observed at pH 6.4 (334), but the extent of coupling apparently increases with pH up to approximately pH 11 (normal sodium carbonate) (331). In many experiments an excess of sodium carbonate was used, the high alkalinity of which would certainly denature some proteins. The loss of immunological specificity of serum proteins on standing at pH 11.0 (17) may very well be due to denaturation. Every effort should be made, therefore, to confine the coupling to the region of pH 7.0-9.0.

Reiner and Lang (14) have noted a difference in color as well as intensity of the coupled compounds of insulin depending on whether coupling took place at pH 7. in the presence of phosphate or in dilute alkali pH 8-9.

Some idea of the very wide range of molecules which have been coupled to proteins can be gained from a few examples. In some instances the protein-coupled complexes have interesting clinical and pharmacological properties. The variable or R portion of all these complexes has in general been attached to the para position in the diazonium salt, though in specific instances ortho and meta positions have been used. The R may be simple aliphatic or aromatic acids, ethers, alcohols, etc. (1), cationic or anionic radicals (335), or they may be such substances as morphine or strychnine (336), amino acids (337), polypeptides (338), cholesterol (339), androstanediol (340), histamine (341, 342), adrenalin (343), aspirin (344), quinine (345), thyroxine (346), optically active sugars (347), and even type-specific polysaccharides of some of the pneumococci capsules (348).

## 11. Miscellaneous

*a. Reaction with Oxazolones.* Lettre and Haas (13) have recently found that oxazolones will react with proteins as indicated below. The



concentrations of O-methyl iso urea hydrochloride and found no indication that a reaction had taken place. Since no mention was made of pH, it may be presumed that the guanidinating action is a property of the free base. Christensen (163) has recently shown that methyl iso thio urea combines with the  $\delta$ -amino groups of ornithine in the polypeptide tyrocidine. Schutte (376) has also found that the S-methyl iso thio urea combines with amino groups. In this case the amino groups were on casein and lactalbumin.

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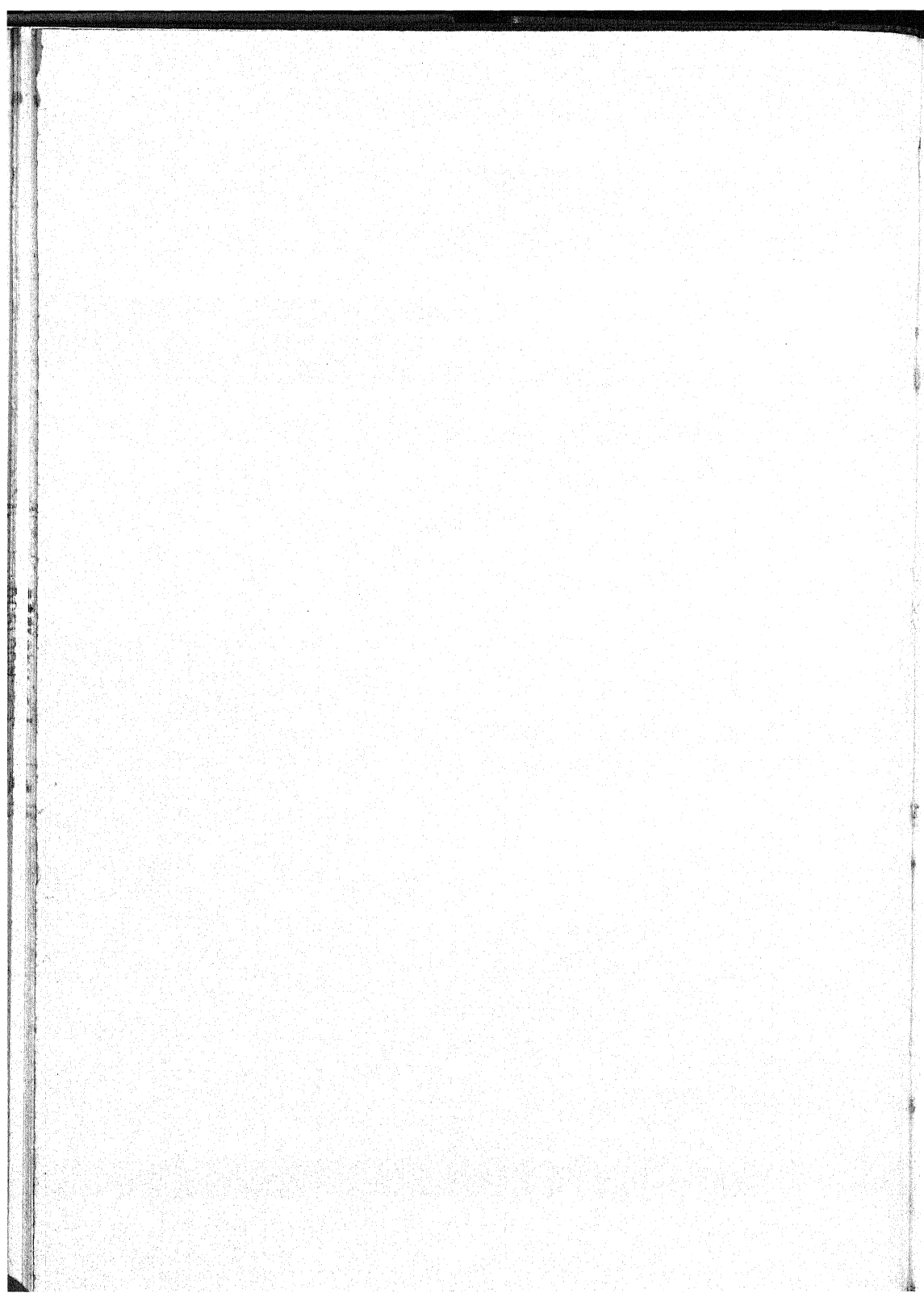


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# The Amino Acid Requirements of Man

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## I. INTRODUCTION

Until recently, the nitrogen requirements of man have been assessed primarily in terms of whole proteins, but with increasing knowledge of the biological behavior and composition of various proteins, it has become evident that nutritional value of the proteins is a characteristic of their component parts, the amino acids. The proteins are composed of twenty or more amino acids; some, the dispensable amino acids, are synthesized in the animal body at a rate commensurate with the needs of the organisms; while others, the indispensable amino acids, are not and must therefore be adequately supplied by the diet. This classification of the amino acids is due to the work of Rose and his associates (1), who established (a) that nine of the amino acids are absolutely required for growth of the immature rat, (b) that in the absence of arginine growth will occur

at a subnormal rate, and (c) that while cystine is not essential, its presence in the diet reduces the methionine requirement by one sixth (2). This last observation demonstrates the fact that unessential amino acids may spare essential ones; many more examples of this have recently been isotopically demonstrated (3). The possibility of a qualitative species difference in the essential amino acid requirements has been given proof in the finding of Almquist (4) that the growing chick needs both glycine and arginine for normal growth. That the amino acid requirements differ even for growth and maintenance has been reported by Burroughs, Burroughs and Mitchell (5) who have been able to maintain adult rats in nitrogen equilibrium for short periods with a mixture of threonine, isoleucine, tryptophan, valine, methionine, tyrosine, and norleucine as the sole source of nitrogen. In addition to these variables, it appears evident that the amino acid requirements for the reproductive function may differ from those of the adult animal. Lysine (6) and tryptophan (7) have been found to be essential for a normal estrous cycle in the rat, and tryptophan has been demonstrated to be necessary for reproduction in the rat (8). The need for arginine for normal spermatogenesis in the rat has also been reported (9), but has not been confirmed (10).

These instances serve to call attention to the fact that the list of essential amino acids established by Rose is based on one function and one species, namely growth of the immature rat, and does not preclude variations arising from other physiological needs of the same or other species. Thus, it may reasonably be assumed that in man, like the rat, no fixed amount of protein will serve to satisfy the needs for all physiological processes of life, growth, adolescence, adult maintenance, reproduction and senescence. Since direct data on the amino acid requirements of man are available only for the adult male and female, the requirements for the other life categories can be deduced only from calculations of nitrogen balance data derived from experiments employing proteins of known amino acid composition. Such deductions from more complex diets are complicated by the fact that (a) the requirements of a single amino acid are a function of the amino acid contour of the protein itself and (b) the requirements may change when quantities of other substances metabolically associated with the proteins are altered. Recently, it has become increasingly evident that the nutritive quality of the proteins is dependent not only on the distribution of essential and unessential amino acids in the protein but also on the content of streptogenin, a polypeptide-like substance such as the streptogenin isolated by Woolley (11). The feeding of normal or even excessive quantities, of good proteins in the absence of adequate quantities of carbohydrate or fats is known to result in a loss of nitrogen in man (12) and in experimental animals. In fact,

it has been shown (13) that within certain limits the protein requirement itself can be considerably lowered by the generous administration of carbohydrates. This "protein sparing action of the carbohydrates" has been found to be equivalent to 1.4 g. of N per day per 200 g. of sucrose in the adult man and 0.25 g. of N per day per 40 g. of lactose in the infant (14). A recent study into the mechanism of this biochemical reaction suggests that the nitrogen sparing resulting from the administration of a single dose of carbohydrate is associated with the retention of tryptophan (15). On an isocaloric basis, the protein sparing action of fats does not appear to be as great as that of the carbohydrates (16). The nutritional inter-relationship of the vitamins and proteins and amino acids is currently being explored with considerable success (17).

## II. THE NITROGEN BALANCE METHOD

The nutritional inadequacy of diets for the human and experimental animals has been commonly ascertained by three criteria: (a) inhibition of physiological functions, namely growth; (b) establishment of negative metabolic balance for the element tested; and (c) induction of specific clinical symptoms or morphological changes. Although it might be expected that with a prolongation of the dietary deficiencies these changes would follow each other in the given sequence, this is often not the case.

### 1. *Relationship of Nitrogen Balance and Body Weight Changes*

In studies on protein metabolism, nutritional adequacy of the nitrogen component in the growing or mature organism is commonly measured in terms of weight change and nitrogen balance. In spite of reliable experimental evidence to the contrary, it has been assumed by some that these two metabolic functions should vary in direct proportion. Such a view fails to acknowledge the obvious possibility that tissue water and fat depots can be mobilized independently of body proteins. Thus, in 1905 Folin (18) observed that in his adult subjects body weight remained constant, and in one instance increased, in the face of considerable losses of urinary nitrogen. Since his data further indicated that the maintenance, or increase, of weight could not have been due to a compensatory accumulation of fat, he attributed the discrepancy to a retention of water and pointed out that changes in body weight occurring in the course of feeding experiments with normal persons may be largely due to gains or losses of water which may or may not coincide with the loss of nitrogen. The converse circumstance, the maintenance of nitrogen balance or even nitrogen retention with a concomitant loss of body weight has also been observed (13, 19).

The possibility of these divergences of weight change and nitrogen balance finds additional support in metabolic studies on infant children.



According to Rubner's estimate, one gram of nitrogen is equivalent to 33 g. of body substance composed largely of muscle. However, in the infant (20) and preadolescent child (21) observed body weight accretions are usually only 50 to 60% of the values calculated from the observed N-retentions

$$\text{g. N-retention} \times 33 = \text{estimated body weight gain.}$$

This discrepancy indicates that composition of weight gain differs in quality, sometimes it may predominate in fluids and other times in fat or protoplasmic tissues. This discrepancy also raises questions as to the function and metabolic fate of the retained nitrogen which fails to appear as body tissue.

## 2. *Factors Effecting the Nitrogen Balance*

The limitations of the nitrogen balance method as a tool for measuring the biological value of experimental diets have long been known and should be constantly borne in mind when interpreting nitrogen balance data. Here, as in all metabolic balance studies, the value of the results is directly proportional to the duration of the investigation. The experience of others and our own, leads to the conclusion that results based on periods of less than 5 days duration are of doubtful value (21, 22). The validity of data secured from test periods of this minimal or longer time can obviously have no significance without pre- and post-control periods of five days or more. The practical implications of such studies can be further increased by adjustment of caloric distribution and intake to simulate that of normal diets. The use of high caloric diets has the tendency to minimize or even obscure defects of the protein moiety while low-caloric diets will exaggerate the deficiency of the protein component. Since it has been shown that nitrogen equilibrium can be established over a wide range of nitrogen intake by a gradual diminution or augmentation of protein fed, it is imperative that the protein content of the experimental diet should closely approximate that of the normal diet. In addition to this source of error, the influence of the nitrogen history of the organism on its reaction to the test diet must be considered (23). This phenomenon has been most clearly demonstrated by McCollum (24) who observed that minimum N-expenditure of pigs on a protein-free diet was considerably lowered by the feeding of zein instead of urea in the fore-period.

In studying the nutritional value of proteins and protein preparations, it is of the utmost importance that the diet be free from other defects. Diets providing a sufficient amount of protein but poor in calories fail to maintain N-equilibrium and weight. Owing to the uncertainties regarding the B vitamin complex, it is preferable to employ brewer's yeast

instead of the synthetically available components of this group, in the knowledge that the amino acid contribution from the yeast can be readily ascertained from existing analytical data (25). Sufficient amounts of water and salts, especially those of calcium and phosphorus must also be uniformly provided throughout the test, pre- and post-control periods. Ingestion of diuretic agents has been shown to lead to false N-balances (26). The errors introduced in nitrogen balance values by the use of racemate containing mixtures of the amino acids cannot be evaluated therefore until the diuretic and other physiological properties of the unnatural forms have been ascertained.

### 3. *Effect of Physiological States on the Nitrogen Balance*

Even though great care be taken to control the conditions of a nitrogen balance study, the interpretations of the findings must take into account the physiological status of the organism. Infants three to twelve months of age have been found to lose weight and yet retain N on diets providing only 50% of the normal food intake (27). It is obvious that in this age group, N-retention *per se* cannot be employed as a criterion of nutritional fitness of the diet, but must correlate statistically with the mean N-retention values of the age and weight group under consideration. The use of such a sliding scale should not include the abnormally high N-retentions which can be temporarily induced in infants fed diets of high protein content. The normal protein requirement for this age group can be determined from an estimate of the N-utilization (N-retention/N-intake  $\times 100$ ) which should not fall far below the N-retentions given below from a review of the literature. The results obtained by Macy and associates (28) in a large series of observations indicate that even for long continuous metabolic periods, the nitrogen balance method for determining protein requirement of children (4 to 10 years) is an inadequate measure since there are factors other than food that are playing determinate roles in nitrogen metabolism of growth. This group of investigators (21) also found that N-retention was only slightly influenced by body weight and even less by surface area. A depression in the retention of nitrogen has been observed to occur with sexual maturation of girls (29) and boys (30). In the adult female, the nitrogen requirements have been observed to decrease with the onset of menstruation (31). It is obvious that the protein requirements be elevated during pregnancy and lactation. The relatively stable physiology of the adult male recommends him as the ideal test subject for nitrogen balance studies. The effect of old age on the stability of the nitrogen equilibrium does not appear to have been determined.

#### 4. *Analytical Details of the Nitrogen Balance Method*

In addition to these precautions, attention is called to the analytical details of the nitrogen balance method which has been amply discussed by Peters and Van Slyke (32) and Macy (21). Unfortunately, two experimental malpractices persist despite contra-indicative evidences: (a) estimation of fecal N by calculation as 10% of urinary N rather than by direct measurement, and (b) use of daily creatinine output as a criterion of adequacy of 24-hour urine collections. Examination of the nitrogen balance protocols of numerous investigators and our own data on 30 normal adult male and female subjects studied for periods of 38 to 60 days discloses that the observed fecal N varies from 5 to 20% of the urinary N and that the median figure is unpredictable even for the same individual for successive periods. The range of variation appears to be greater for the adult than for the infant or growing child. Obviously, conclusions based on results derived from the application of the "10% rule" must be accepted with serious reservations. Although the general validity of Folin's law of constant daily creatinine has been generally accepted, the constancy which it asserts must be allowed to be something short of absolute, as is indicated by the data upon which it is based (33). In an extended study on 33 normal and female subjects on hospital fare, Wang (34) found fluctuations of 10 to 25% of the individual creatinine output to be of common occurrence. In our experiments (35) individual daily variations of 10 to 25% of the total creatinine were also observed. These observations would seem to indicate that variations in creatinine output are not necessarily indicative of inaccurate 24-hour urine collection.

These shortcomings of the nitrogen balance method led us to attempt a supplementation of these data by measurements of the urinary amino acids and other metabolites. It is interesting to note that the urinary bound amino-N-output has been found to characterize the infant metabolism of various milk protein preparations more significantly than the N-retention values (36). This approach as will be discussed later has also afforded us a supplementary means of ascertaining the human requirements of tryptophan, lysine, and methionine; and a measure of the dietary lack of amino acids, histidine and cystine, which fail to induce negative N-balance.

In conclusion, it may be said that the N-balance is probably the most valuable single criterion of the adequacy of the nitrogenous moiety of the diet. It is, however, subject to certain errors which have been discussed and which must be taken into consideration when interpreting the data.

### III. PROTEIN AND AMINO ACID REQUIREMENTS OF MAN

The estimation of the individual amino acid requirements of man constitutes a recently opened field of biochemical inquiry. Its advance

is hampered by the incomplete understanding of the protein needs of the human at various life periods. The survey of the literature which is presented here reveals the startling insufficiency of data upon which studies of specific amino acid requirements must be based and points out the need for a systematic filling in of these gaps.

### 1. *First Year of Life*

In attempting to assess the protein needs of the growing child, we do not have a fixed criterion of protein adequacy, namely N-balance to go by. We have to judge by the rate of N-retention. Unfortunately, exact criteria for normal retention at different ages are not applicable. Wide variations of 100% or more are encountered in the data recorded in the literature. These are due to several factors, chief among which is the previous nutritional state of the subject. An undernourished child will during convalescence retain extraordinarily large amounts of protein. Apart from preceding illness, diets vary in their ability to induce storage of reserve protein (see p. 261). If the experimental diet is more conducive to this than the preceding diet, high N-retentions will be observed and *vice versa*. It is usually accepted that the increased N-retention brought about by increasing the N-intake is a relatively temporary affair lasting a matter of weeks only after which the original retention level is resumed. Nevertheless observations have been presented by Nelson (42) that some increase of N-retention continues indefinitely, resulting in a bigger, but not necessarily a better subject. Further long range observations are needed to establish this last point.

Bearing in mind these limitations in interpreting N-retention data in growing subjects, all that can justifiably be done at the present time is to point out a fairly wide range of normal retentions at different ages.

Since the amino acid composition (37) and caloric distribution of various milks are reasonably well established, the amino acid needs of the premature and full-term infants through the first year of life can be determined approximately from their milk intake. The nitrogen requirements of premature infants younger than 3 weeks (1600 g.) have not been studied. Several earlier investigators (38) and more recently Gordon and his associates (39) have presented evidence that one to two months old prematures retain about 250 mg. of nitrogen per kilogram per day on an intake of 360 - 500 mg. of milk protein N per kilogram per day. Gordon also found that at the comparable level of feeding for premature infants no difference could be detected between the nitrogen retention by infants receiving modified cow's milk and that of infants receiving human milk for periods as long as two weeks. The plotting of Gordon's data as shown in Fig. 1, indicates that nitrogen intakes of less than 450

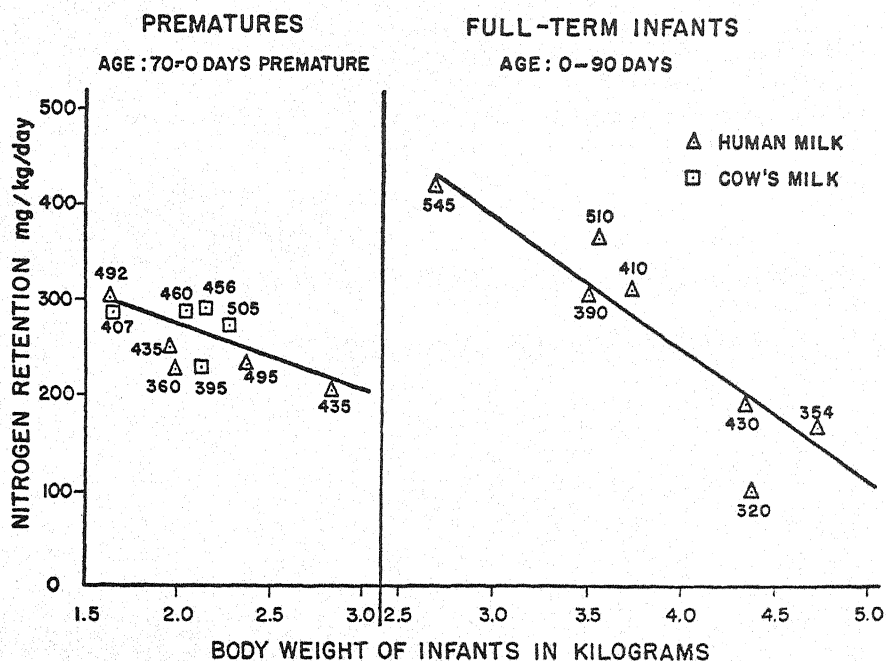


FIG. 1. Relation of N-retention to milk protein N-intake and body weight of premature and full-term infants. The chart was constructed from the data of Gordon on prematures and the data given by Czerny and Keller (40) on the full-term infants. They represent feedings of modified cow's milk and the feeding of human milk. The figures inscribed about these signs denote milligrams of N-intake per day per kilogram of body weight.

mg. of N and higher than 500 mg. of N per kilogram of body weight result in retentions which are respectively below or above the mean, thereby suggesting that 475 mg. of milk protein N per kilogram of body weight per day adequately fulfills the protein requirements of the premature infant. Inasmuch as the mixtures of cow's milk were modified by the addition of dextrimaltose, olive oil and water so that they approximated the human milk in protein, fat, carbohydrate and fluid content, the only remaining variable resided in the amino acid composition of the protein moiety. Since human milk used in these experiments contained an average 1.44 g. protein per 100 cc. or 230 mg. of N per 100 cc., it would be necessary to feed 206 cc. of human milk to attain the apparent required intake of 475 mg. of N per kilogram per day. Calculations from these figures and the data on amino acid content of human and cow's milk reported by Williamson (37), it becomes possible to estimate the amino acid requirements of the premature per kilogram per

day (Table I). These values, of course do not represent minimal quantities but give a safe estimate of the range of the needs of the individual amino acids.

TABLE I

*Daily Amino Acid Intake for the First Year of Life*  
*Calculated from Milk Protein Requirements Necessary for Optimal Nitrogen Retention\**  
 Milligrams of amino acid per kilogram of body weight

Amino Acid	Prematures	Full-term Infants	
	Modified Cow's Milk	Human Milk	Modified Cow's Milk
	70-90 days premature	0-90 days	3-12 months
Alanine	76	59	89
Glycine	12	10	14
Proline	256	134	300
Glutamic Acid	695	384	820
Aspartic Acid	170	194	199
Serine	164	115	192
Threonine	156	105	183
Leucine	504	380	590
Isoleucine	170	125	199
Valine	176	111	203
Cystine	29	69	34
Methionine	103	49	120
Tyrosine	176	122	204
Phenylalanine	188	129	210
Histidine	66	42	78
Arginine	131	112	154
Lysine	205	157	240
Tryptophan	44	52	58

\* These figures are undoubtedly above the minimum requirements for the particular amino acid in question and it might be possible to reduce the given values for non-essential amino acids to zero. It is obvious from the available data that provided all the requirements for essential amino acids are met, the remainder necessary to make up the total required quantity could be distributed among different types of amino acids with wide possible variations, all of which might be of equal nutritional value. Of course, we must always bear in mind the possibility that the non-essential amino acids may become essential by virtue of a specific nutritional need or by their sparing action of some essential amino acid; e.g. the cystine-methionine relationship.

Similar mathematical treatment of available nitrogen balance data (21) for the first 90 days of life of the full-term infant of normal birth weight (Fig. 1) indicates that an intake of 400 mg. of human milk nitrogen is required for normal nitrogen retention which again appears to be a function of weight rather than age (40). An approximation of the specific amino acid needs of the infant of this age group based on the mean

TABLE II  
*Relation of Milk Protein Nitrogen Intake to Nitrogen Retention of the Infant*

AUTHORS	Daniels and Hejman (41)				Nelson (42)				Jeans and Stearns (43)			
	Dextrin-maltose modified cow's milk				Acidified undiluted cow's milk				Acidified diluted evaporated milk			
	No. of Subjects	Body Weight	Intake	Nitrogen Retention	No. of Subjects	Body Weight	Intake	Nitrogen Retention	No. of Subjects	Body Weight	Intake	Nitrogen Retention
WEEKS		kg.	mg./kg.*	mg./kg.*		kg.	mg./kg.*	mg./kg.*		kg.	mg./kg.*	mg./kg.*
5-10	1	5.40	497	113	5	5.18	688	211	7	5.44	640	179
10-15	8	5.83	535	168	16	6.26	652	185	13	6.24	599	143
15-20	12	6.66	525	136	15	7.26	580	170	15	7.06	589	147
20-25	6	7.70	525	149	9	8.07	581	146	13	8.07	579	193
25-30	5	7.89	565	160	8	8.39	600	147	12	8.28	593	186
20-35	2	9.77	485	86	6	9.09	582	154	8	8.64	604	201
35-40					5	9.59	550	144	9	9.65	530	113
40-45					7	10.49	530	123	8	9.86	495	139

\* mg. per kg.



required N-intake is also given in Table I. Comparison of the N-retention curves of the two groups reveals a greater gradient for the full-term infant than for the premature infants suggesting a more efficient N-utilization in the prematures at normal levels of protein intake.

Numerous determinations have been made of the protein nitrogen needs of infant, 3 to 12 months of age, employing human milk and a variety of modifications of cow's milk. Some of the available data have been recalculated to a uniform base and are listed in Table II. The N-retention values fail to indicate any nutritional advantages of the different modifications. Although it is not evident from Table II, examination of the original data reveals that the quantity of nitrogen stored tends to increase with well tolerated increases in milk intake. This phenomenon is not only apparent for short periods of time but has also been shown by Nelson (42) to lead to the development of larger but not better babies. No doubt the maximal retention which can be affected in this fashion must be limited in magnitude and duration. Jeans and Stearns (43) observed that the retentions of nitrogen were larger below 20 weeks of age in the group fed fresh milk and between 20 and 35 weeks in the group fed evaporated milk. The observed lower nitrogen retentions of the early weeks were thought to be due in part to gastro-intestinal disturbances incident to over feeding rather than to any deleterious properties of evaporated milk. With due consideration to these restrictions on the validity of the N-retention figures, it appears from Table II, that 500 - 600 mg. of cow's milk protein nitrogen per kilogram of body weight must be fed infants of this age group, which is somewhat higher than the average 475 mg. of protein N fed the prematures and new borns by Gordon (39). The individual amino acid equivalent of the protein requirement for the age group 3 to 12 months is given in Table I. Shohl and coworkers (44) have reported that the N-retention of infants 2 to 7 months of age was not altered by substitution of a synthetic formula composed of amigen, carbohydrates and fats for an evaporated milk formula when they were fed at the same fluid caloric and nitrogen levels. Nitrogen intakes from these formulae of 520 mg. of N per kilogram resulted in nitrogen retentions of 140 mg. and 170 mg. of N per kilogram, respectively. It will be noted that these values are in fair agreement with those listed in Table II. In contrast to these observations, Beach and associates (45) have reported that a growth of 25 g. per day was attained by two infants 1 to 5 months of age on an intake of 300 to 200 mg. of nitrogen per kilogram which was derived solely from human breast milk.

Previous reports (46, 47) from this laboratory indicated that diets prepared with mixtures of the crystalline amino acids or acid hydrolyzates



of the proteins exhibited about 50% of the growth coefficient in the immature rats as that of diets constructed from enzymatic digests of the proteins or whole proteins. Subsequent work by Woolley (11) has shown that the optimal growth characteristics of diets prepared from whole, or enzymatically digested, proteins are due to a peptide-like substance occurring in these products.

In order to evaluate the significance of this new growth factor in human nutrition, normal infants, 3 to 12 months of age, were fed synthetic formulae composed of protein digest, fats, carbohydrate, brewer's yeast, salt mixture and water in such proportions as to simulate the caloric distribution and mineral content of cow's milk as commonly modified for infant feeding. These diets were given in five feedings daily at the rate of about 100 calories per kilogram of body weight which were supplemented daily with 50 mg. of ascorbic acid and 15 drops of oleum percomorphum. The individual protein preparations were tested for seven day periods and the biological value of the preparations during this interval measured in terms of body weight change, nitrogen retention, blood protein levels and urinary amino acid output.

These experiments disclosed that the N-retention and weight gains of the infants on a tryptophan and cystine reinforced acid digest of casein were inferior to those obtained when enzymatic digests of casein or lactalbumin were employed as the protein component of the formulae. The nutritional values of the enzymatic products were slightly inferior by these criteria to an evaporated milk formula providing a similar distribution of an equivalent number of calories and an equal amount of whole protein. The blood protein levels failed to show any significant variations with shifts of the nitrogen component of the diet. These findings suggest that an acid labile and enzyme stable, probably streptogenin-like substance occurring in native proteins is also required for optimal growth of the human.

The urinary amino nitrogen data obtained in these experiments, revealed that the bound amino nitrogen output on administration of the acid hydrolyzate preparation was twice as great as that occurring with the other protein preparations. This greater output of polypeptide nitrogen suggests that the poor weight gains observed with acid digest diet were due in part to a loss of nutritional efficiency of the organism incurred by an accelerated catabolism of tissue protein, for the purpose of making available components required for the more indispensable body proteins (48).

Although clinical investigations (49) have claimed that human milk has nutritive advantages over other types of infant foods, the available nitrogen balance data fail to support this contention. The claimed

nutritive virtues of human milk do not appear to reside in the vitamin or ash phases. Differences in the amino acid contour of the proteins of cow's and human milk have been the source of much unwarranted speculation. Since the protein content of cow's milk is almost twice that of human milk, it follows that cow's milk should contain larger amounts of all the amino acids. However, there are noteworthy differences between human milk and diluted cow's milk as commonly used. Thus, although human milk contains three times as much cystine as does modified cow's milk, cow's milk as ordinarily diluted for infants, formulae proteins provides  $1\frac{1}{2}$  times as much methionine as do the human milk proteins. The lower methionine content of human milk probably does not represent a nutritive deficiency since the large amount of cystine may reduce the methionine requirement. However, on the basis of millimoles of total sulfur amino acids, there is no significant difference between the two milk sources. It is to be further noted that diluted cow's milk contains greater quantities of valine, threonine and histidine, while human milk is richer in tryptophan (37). None of these differences seems to be of such magnitude as to support the clinical impressions of the nutritional superiority of human milk.

## 2. Preadolescence

Owing probably to technical difficulties, the nutritional requirements of the child from 1 to 3 years of age have not been measured by the N-balance technique. In contrast, numerous metabolic studies on children above this age have been reported; the most complete being that of Macy and her associates (21). The data on nitrogen metabolism abstracted from this work and that of others are shown in Table III. In general these protein intake values are somewhat lower than those recommended by Holt and Fales (55). The large standard deviations for the average retentions for each age and body weight group lead Macy and coworkers to doubt the usefulness of adjusting protein intake to either of these two variants. These authors feel that the relationship of nitrogen retention to nitrogen intake is a more significant index of nitrogen metabolism of the child and adequacy of the diet than any other mathematical correlation of nitrogen balance data. They conclude from their investigations that an average daily consumption of 473 mg. of nitrogen per kilogram of body weight per day is adequate for all children of this age group. Although it is obviously difficult to determine the amino acid composition of a heterogeneous diet composed of animal and vegetable proteins, a tentative estimate of the amino acid content of the diet of a 12 year old child has been made by these workers and is compared in Table IV with the amino acid content of an equivalent amount of casein

TABLE III  
*The Protein Nitrogen Intake and Retention of the Preadolescent*

Authors	No. of Subjects	Age	Average Weight	N-Intake	N-Retention
		yrs.	kg.	mg./kg.	mg./kg.
Porter (50)	1	2.5	15	428	87
	1	4.7	18	374	64
	1	5.5	23	339	54
Daniels (51)	1	3.0	19.5	454	50
	1	4.0	19.5	500	56
Daniels (52)	8	4.4	15.4	550-590	121
	17	4.4	16.2	500-540	101
	15	4.7	16.9	450-490	83
Wang (53)	13	7.2	—	516	89
	10	9.9	—	448	42
Wang (54)	7	8.9	25.8	537	76
	7	8.9	24.9	264	1
Macy (21)	5	4	18.4	522	37
	5	5	18.7	520	32
	6	6	21.8	466	20
	6	8	26.2	442	24
	2	9	28.4	457	37
	3	10	33.3	393	31
	1	11	35.8	371	22
	1	12	42.0	317	19

TABLE IV  
*Comparison of the Estimated Daily Amino Acid Intake of a 12-Year-Old Boy on a Mixed Diet and the Amino Acid Content of an Equivalent Amount of Casein*

Amino Acid	Natural Food Diet Data from Macy (21)	Casein (61) Data calculated by the author
	g.	g.
Cystine	0.94	0.26
Methionine	2.35	2.54
Arginine	4.26	2.97
Histidine	1.41	1.82
Lysine	4.16	5.00
Tyrosine	3.46	4.65
Tryptophan	0.83	1.30
Phenylalanine	3.78	3.78
Threonine	2.87	2.83
Valine	2.83	5.04
Leucine	8.65	8.76
Isoleucine	2.77	4.72
Total Amino Acids	38.31	43.67
Total Known N-Intake	11.63	11.63

nitrogen. It appears from this tabulation that the amino acid intake provided by the proteins of natural food diet is not very different from an equivalent amount of casein.

As might be expected the N-retention of the growing child is seriously affected by dietary factors other than the quantity and quality of the protein component. Chaney and Blunt (56) found that N assimilation of two growing girls, 10 and 11 years old, on diets of comparable protein content was augmented three fold by the daily administration of 600 to 700 cc. of orange juice. In view of the recently observed metabolic relationship of vitamins and amino acids in man and experimental animals (17), these noteworthy experiments should be repeated and supplemented with data on urinary metabolites. Wang and associates (53) reported that the per cent nitrogen retention of children 6 to 13 years of age was greater for those underweight than those of normal body weight. These workers also observed (54) that the nitrogen retention of nine undernourished children was greater than that of eight normal children of similar age, 4 to 12 years, on a low but not on a high protein intake. Hubbell and Koehne (57) in a study of 17 children from 7 to 11 years of age found that the average retention of 26 mg. of N per kilogram per day was not appreciably altered by the inclusion of sugar to give a 6% increase in caloric value of the diet. Davis (58) found that the nitrogen retention of children 8 to 11 years of age was higher on a base-forming diet than acid-forming diet, even though the protein intake was slightly greater with the latter diet. Administration of viosterol to girls of premenarchial age has been shown by Johnston (29) to significantly increase the N-retention. The nitrogen retention of children 6 to 8 years of age has been reported by Nothass and Schadow (59) to be increased under the influence of sea climate with no increase in body weight. Ultraviolet irradiation has an irregular effect. Thyroid administration was found by Johnston and Maroney (60) to increase N-retention.

There appears to be no marked sex differences in N-retention or protein requirements for this age group (21, 55).

### 3. Adolescence

The subject of the protein intakes of adolescent boys and girls was investigated and reviewed by Holt and Fales (55) in 1921 and comparatively little has been done since that time. The data from their study of healthy children, 10 to 17 years of age, from intelligent well-to-do families are shown in Fig. 2.

It is to be noted that the protein intakes, consisting of two thirds animal protein and one third vegetable protein, are generally higher for

the boys and especially so in the tenth, thirteenth and fourteenth years. Since the protein intake in these studies was regulated by appetite alone, the values do not represent minimum figures. However, the accord of these protein values with those of Wait and Roberts (62) for adolescent girls which are based on N-balance data indicate that appetite constitutes a good criterion of this dietary need. The report of Wait and

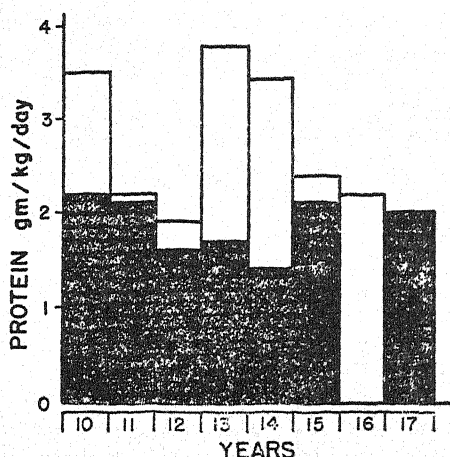


FIG. 2. Comparison of protein intake of adolescent boys and girls. This chart was constructed from the data reported by Holt and Fales (55). No suitable values are available for boys in the seventeenth year and for girls in the sixteenth year.

Roberts which reviews the earlier literature and presents their own data on 52 healthy girls from 10 to 16 years of age, shows that of the 13 balances on normal children of 10 to 11 years, all intakes below 2 g. of protein per kg. resulted in a loss of nitrogen from the body while storage was achieved on protein intakes of 2.57 to 3.08 g. per kilogram. These investigators found no close relation between the required total protein and any of the single variants, age, weight and height. When combinations of three rather than merely two of these bases are used, the correlations are higher, with the exception of height referred to age, and the calculated figure closely approximates the observed results. Recently, Johnston (19) has found that sexual maturation in girls, which is not necessarily coincidental with the onset of menstruation, carries with it a depression in the retention of nitrogen. Failure to increase the dietary protein intake which is indicated by this fall in N-retention favors the spread of tuberculosis in girls with minimal reinfection type of lesions.

Since the Holt and Fales publication, only one report, that of Hsu and Adolph (63), has appeared on the protein requirements of adolescent

boys. In this investigation with two groups of boys, 14 to 16 years of age, one fed on a white bread diet supplemented with dried cow's or soybean milk and the other group maintained on a mixed cereal diet, they found that the nitrogen retention could be raised from 50 mg. of N per kilogram to 80 mg. of N per kilogram by increasing the protein intake from 1.68 to 1.90 g. per kilogram. This indicates that the lower protein intake was suboptimal.

It is unfortunate that so few data are available on the protein metabolism of the human at this critical age.

#### 4. *The Adult*

Numerous and conflicting reports on the protein requirements of the adult are to be found in the literature (23, 64, 65, 66). The use of heterogeneous natural food stuffs in the majority of these studies does not permit a ready estimation of the human amino acid requirements from the nitrogen data. In balance studies in which milk proteins constitute the principal nitrogen component of the diet, it is possible to approximate

TABLE V

#### *Amino Acid Requirements for Adults to Maintain Nitrogen Balance*

Milligrams intake per kilogram of body weight

Amino Acid ("Natural Forms")	Casein Authors	Milk Proteins	
		Martin and Robison	Rose and McLeod
Glycine	3.1	2.70	1.84
Alanine	11.2	12.9	8.8
Valine	49.4	47.6	32.5
Leucines	60.1	70.3	48.0
Aspartic Acid	25.6	33.9	23.1
Glutamic Acid	136.3	129.0	88
Serine	31.2	29.8	20.3
Threonine	22.0	24.8	17.0
Proline	50.0	48.7	36.5
Hydroxyproline	2.5	2.6	1.7
Phenylalanine	29.4	23.6	16.1
Tyrosine	37.5	41.9	28.6
Cystine	7.4	4.9	3.3
Methionine	18.5	22.1	15.5
Tryptophan	9.4	9.3	6.2
Arginine	22.6	27.0	18.4
Histidine	19.9	12.6	8.6
Lysine	37.5	47.1	32.1
Caloric intake per kilogram	43.5-40.0	54	40
Body weight per kilogram	55-100	—	54
Sex	males and females	males	females

the amino acid needs of the adult. In the nitrogen balance experiments of Martin and Robison (23), of M. S. Rose and MacLeod (65) and the data from the control periods of our human amino acid deficiency studies, in which milk proteins constitute the principal nitrogen component of the diet, it is possible to approximate the amino acid needs of the adult. The results of computations from the data from these three sources and the published analytical data of these proteins (61) are given in Table V.

In comparing these amino acid values, it is important to note some experimental and dietary and other differences of the investigations. Martin and Robison employed a purified diet with whole cow's milk providing about 95% of the dietary nitrogen. The experiments of Rose and MacLeod were essentially similar except that apples were fed in addition to the purified foods and milk. In our work, the 100 mg. of N per kilogram per day fed the subject was derived from an enzymatic digest of casein or from an acid hydrolyzed casein concentrate enriched by 1.5% of *l*-tryptophan and 1.0% of *l*-cystine of the protein content (calculated as  $N \times 6.25$ ). When this amount of nitrogen was given with a total supplement of approximately 800 calories of fat and 1500 calories of carbohydrate, N-balance was achieved and sustained without loss of weight in 30 subjects (5 females; 25 males) varying in body weight from 55 to 104 kg. for periods of 10 days. The non-protein portion of the diet consisted of natural fats and carbohydrates and fruits and vegetables selected for their low nitrogen content and contributed an additional 5 to 10% of the total N of unknown amino acid content. The B complex vitamins were supplied by three tablets per day of yeast concentrate and the other vitamins were provided by the natural foods.

W. C. Rose and coworkers (67) were able to maintain N-balance in young men with high caloric diets constructed from racemate containing mixtures of nine crystalline amino acids, purified fats, carbohydrates and vitamin mixtures. Since the nine essential amino acids of those diets had to be converted in part to the ten unessential amino acids, the requirement of each of the nine essential amino acids appears to be about twice that shown in Table V.

In order to obtain exact information about the minimal amino acid requirements, we have recently initiated studies in which diets deficient in a single amino acid were supplemented by the stepwise addition of the missing amino acid so that the minimal intake that would restore N-equilibrium could be determined. By this means, we found (68) that restoration of nitrogen equilibrium occurred with a supplement of 6.0 to 9.0 mg. of tryptophan per kg. per day. However, a tryptophan supplement of only 3.0 to 6.0 mg. per kg. was required to restore urinary tryptophan output to normal levels.

A statistical study of the per capita protein consumption by Block (61) shows that the amino acid intake of the average American is far above the values listed in Table V.

### 5. *Pregnancy*

The need for increasing the protein intake of women during pregnancy would seem to be an obvious dietotherapeutic measure. Yet prior to 1936, it was the practice of most obstetrical authorities to restrict the diet for pregnant women, especially those with toxemic tendencies, in regard to animal proteins. This practice found easy acceptance since a dietary survey by Barker (69) disclosed that many women avoided eating meat and other protein-rich food in fear of getting fat or developing too large a baby. Contraindications to this regimen came from the observations of Dodge and Frost (70) and others who noted that patients who disobeyed orders and ate meat seemed to do as well as, if not better than, patients who observed the restrictions. Accordingly, they began to prescribe six eggs a day in addition to 1 or 2 quarts of skimmed milk, legumes and whatever meat the patient could afford, with the result that the patients began to feel much better, some toxemic patients improved dramatically and according to them eclampsia incidence of the clinic fell to zero as compared to the several cases occurring annually during the animal protein restriction era. More recently Holmes (71) surveyed the incidence of toxemia in 1400 pregnant patients, 700 of whom (primiparas and multiparas) received a low but adequate protein diet and 700 of whom received comparatively high protein diets. He found that the incidence of toxemia in the low protein group was almost twice that in the high protein group.

The need for augmenting rather than diminishing the protein intake during pregnancy had been indicated prior to these clinical impressions by studies on blood protein levels during pregnancy. Thus during the first six months of pregnancy, the serum proteins slowly fall from 7 g. to about 6.2 g. per 100 cc. (32). In the last trimester, they rise almost to the normal values, drop again following delivery then gradually rise and reach the normal non-pregnancy level about 7 days post-partum (72). Eastman (73) had also noted a decrease in total protein during gestation with a slight relative decrease in albumin-globulin ratios in normal pregnancy, 1.7, and better-defined absolute increase in pre-eclampsia, 1.3, and eclampsia, 1.6. In cases with persistent albuminuria, the total serum protein fell to 4.0 to 5.0 g. per 100 cc. which led him to conclude that the resultant decrease in osmotic pressure might play a role in the production of edema in the toxemias of pregnancy. These findings were confirmed by Strauss (74) and Dodge and Frost (70) who made the additional observation that in toxemic patients, the plasma



albumin fell to 3.87 g. % while the globulin rose to 2.07 g. % as compared to a non-pregnant normal of 4.90 and 1.88 g. respectively.

Bethell (75) has found 54% of 158 pregnant women to have true macrocytic anemia which resembles that of pernicious anemia, being characterized by cell counts of less than 3.5 million and hemoglobin concentration of 10 g. %. In most instances, the etiological factors were lack of iron and insufficiency of animal protein.

In view of these clinical and chemical observations, the need for an accurate estimation of the protein requirement of the pregnant woman is obvious. An early report by Coons and Blunt (76) on the nitrogen intake, output and retention of nine pregnant women receiving 8 to 14 g. protein N daily demonstrated that although nitrogen storage had no peak and was unpredictably irregular, there was a tendency to high nitrogen retention at the middle of pregnancy with a decrease in the terminal months. The highest nitrogen retentions occurred in those with the highest protein intakes. Hunscher and associates (77) reported findings which confirm those of Coons and Blunt and interpret their data to indicate that the daily nitrogen retention of the maternal body throughout the pregnancy period are in considerable excess of the needs of the fetus and its adnexa. The maternal nitrogen reserves at full term have been estimated at 100 to 200 g. of N which appear to be required for the nitrogen losses incident to parturition, involution of tissues and initiation of lactation. This group of investigators (78) point out that from the available data, there seems to be no need of increasing dietary protein consumption of pregnant women beyond 70 to 100 g. daily. This range coincides with the level of protein intake (90-100 g.) apparently found necessary by Dodge and Frost to decrease the incidence of toxemia. Further confirmation of these values is available in the results of Harden (79), who sought to maintain pregnant patients in positive nitrogen balance in order to supply the requirements of the fetus without depleting maternal proteins. The protein requirements in toxemia were calculated by adding the amount of protein metabolized, as evidenced by the urinary end products, and the estimated requirements of 2 kilogram fetus to 50 g. daily intake of protein considered to be the minimized maintenance quantity. This investigator also pointed out that the selection of proteins essential in growth and development is necessary to the particular needs of the fetus and storage demands of the maternal host. Harden's 522 patients averaged from 75 to 80 g. of protein daily and none had convulsions, while the frequency of convulsions in referred cases in the locality remained the same. Despite all of the evidence proclaiming the toxemia-preventing effect of adequate protein diets, Bibb (80) has observed the occurrence of hypoproteinemia in toxemic pregnant women on protein intakes which would ordinarily be adequate to maintain good

serum levels, which leads him to believe that some factor other than chronic dietary deficiency must be sought.

The ensuing lactation period has also been estimated to increase the protein requirements (78, 81) to 60% beyond the needs during pregnancy. This is evident from the fact that from 1.0 to 1.5 g. of N are lost daily by the mother through her milk. The intimate relationship of the milk proteins of the nursing mother to those of her diet has been demonstrated by Shannon (82) and others.

### 6. *Senescence*

Almost nothing is known of the biochemistry of aging (83). The metabolism of old age was reviewed by Robertson (83b) in 1907 and few advances have been made since. A study of Fenger (84) upon a woman 61 years of age, weighing 42 to 45 kg., who lived for 15 years upon a diet providing about 2 g. of protein and 25 to 30 kilo *calories* per kg. per day, demonstrated (a) that the ability to utilize food did not seem to change with age, (b) that protein was well digested and absorbed, and (c) that weight and good health were maintained throughout the entire 15 years. Protein balance studies by Koch (85) upon five men, ranging in age from 54 to 79 years of age, fed simple diets of meat, potatoes, and vegetables which provided an average of 10.6 g. of protein-N daily, disclosed that they utilized an average of 86% of the protein. These observations and those of Forster indicate that there is no marked alteration with advancing age in the ability of the body to utilize proteins. The ability of the senile body to metabolize food despite the increasing incidence of achlorhydria after the age of 60, suggests a considerable margin of safety in the mechanisms for food assimilation. Muhlmann (86) observed a marked decrease in excretion of N and S and oxidized S in the course of human aging which leads him to believe that the body lost its ability to absorb protein as age progressed. However, his values fluctuated widely since no attempt was made to control food intake.

The hemoglobin of man tends to remain relatively constant after the age of 11 although there is, according to Williamson and Ets (87), some trend towards a decline in the male after the age of 60. No plasma protein studies appear to have been published for this age group.

The paucity of biochemical information on old age recommends geriatric biochemistry as a field of investigational opportunities, with the nutritional approach probably affording the most promising line of attack.

In concluding this section on the protein requirements of man, we wish to refer the reader to two excellent works on the subject. The classical publication of Martin and Robison (23) which appeared in 1922, reviews the history of attempts to solve the problem of protein maintenance

requirements by means of N-balance data and presents the results of their own metabolism experiments. The noteworthy review of Leitch and Duckworth (88) should also be consulted for a lucid presentation of some fundamental concepts of nitrogen metabolism and for data on the protein allowances recommended by the League of Nations Technical Commission and other nutritional surveys. The protein intakes recommended by the Food and Nutrition Board of the National Research Council, as revised in 1945, are given in Table VI.

TABLE VI

*Daily Protein Requirements Recommended by National Research Council (1945)*

Age or Status	Average Body Weight	Protein
	kg.	g. per day
Children		
Under 1 year	—	(3.5 per kg. body weight)
1-3 years	13	40
4-6 years	19	50
7-9 years	25	60
10-12 years	34	70
Girls		
13-15 years	49	80
16-20 years	54	75
Boys		
13-15 years	47	85
16-20 years	64	100
Women	56	60
Pregnant	—	85
Lactation	—	100
Men	70	70

### 7. Conclusion

It is clear from the foregoing that all of the available data do not permit the establishment of a definite list of protein requirements for the different physiological states of man. The best that can be done at present is to give a list of safe allowances such as have been indicated in Table VI. A comparison of these values with reported results derived from nitrogen balance data reveals them to be generous. However, since the quantity of protein needed is inevitably determined to a large extent by its quality, the difficulties embodied in preparing such tabulations are self-evident. Our knowledge of these matters is in the frontier stages and points to the need of numerous long term nitrogen balance studies complemented with urinary excretion data, which will permit the estab-

ishment of zone if not a precise figure of protein requirements and a list of the relative biological value of various protein foods.

#### IV. AMINO ACIDS ESSENTIAL FOR MAN

In classifying amino acids into dietary essential and unessential groups, it must be borne in mind that the classification was established by Rose on the basis of the growth promoting action of the amino acids in the immature rat, and does not refer to the physiological importance of the essential or unessential amino acids in maintaining normal body functions. Thus, although cystine is generally classified as a dietary unessential amino acid, it is an essential constituent of the plasma proteins, of insulin and of glutathione, and therefore must be considered physiologically indispensable. Examples of these relationships can be repeated for all of the unessential amino acids.

In performing experiments to determine the dietary role of amino acids in the human, several procedures are available for producing diets deficient in single amino acids. Deficient natural proteins used in the pioneer studies of Osborne and Mendel and others, offer only limited and sometimes uncertain possibilities to the investigator. Mixtures of pure amino acids, used with notable success by Rose, permit one to study all possible deficiencies of the known and isolated amino acids, but are not ideal, since the natural optical isomers of certain amino acids are not now obtainable, making it necessary to use racemic forms, the unnatural component of which may act in an unphysiological manner. The degradation of complete proteins or hydrolyzates, by chemical procedures designed to destroy or remove particular amino acids, has the advantage of preserving almost all of the amino acids in their natural form. We have used this approach in preparing our experimental diets. It can be criticized on the ground that the chemical manipulations may injure the diet in unknown ways. The deficient diets were, however, tested on experimental animals with and without supplement of the missing amino acid, and were considered satisfactory only if the supplemental ration restored normal performances in the animal. The particular advantages and disadvantages of the two techniques are summarized in Table VII.

Our experiments have been carried out on healthy adults of both sexes between 21 and 25 years of age, who were leading a sedentary life. They were given weighed diets providing approximately 40 kilo-calories and 0.1 g. N per kilogram per day. These diets were constructed from sugars, starches, fats and certain fruits and vegetables selected for their low nitrogen content, these last supplying about 10% of the daily N-intake. The remaining 90% of the protein moiety consisted of a protein hydrolyzate rendered deficient in a single amino acid. To date, 30 experiments

TABLE VII  
*Comparison of Nutritional Qualities of Protein Hydrolyzates  
 and Crystalline Amino Acid Mixtures*

Criteria	Protein Hydrolyzates	Crystalline Amino Acid Mixtures.
Purity	Virtual freedom from unnatural optical isomers.	Freedom from non-amino-acid impurities.
Utility	Composition readily varied by supplementation, less readily by degradation.	Composition can be readily varied.
Metabolic Stress	Synthesis of unessentials is avoided.	Deamination and excretion of (unneeded) unessentials is avoided.
Toxicity	Toxic effects attributed to unnatural isomers (e.g., phenylalanine) are avoided	Toxic effects attributed to unessentials (e.g., glutamic acid) are avoided.
Speed of Administration	More rapid injection of crystalline mixture may not mean more rapid utilization, because of greater loss in urine.	More rapid injection is possible.
Adequacy	1. Possibility that some "unessential" amino acid may prove essential.  2. Possibility that hydrolyzates may contain an undiscovered amino acid which is essential.	
Availability	Generally available.	Not generally available

with adults on 7 amino acid deficiencies have been carried out, in which deficient periods lasted from 3 to 6 weeks and were preceded and followed by control periods in which a supplement of the missing amino acid was added to the diet. The subjects have been studied clinically and by a number of laboratory techniques for evidences of deficiency, and nitrogen balances were obtained throughout by N-assays of the food and excreta.

Evidence obtained by nitrogen balance studies points clearly to the need for tryptophan (89), lysine (90), and methionine (91) in the diet of man. Each of these deficiencies promptly induces a negative nitrogen

balance, and restitution of the missing amino acid restores nitrogen equilibrium.

The earlier experiments with the cystine-poor diets (91) were inconclusive since the female subject retained her weight and nitrogen balance, while both males lost weight, but only one showed a negative N-balance. In a subsequent experiment (92) two healthy males were given a diet deficient in both sulfur amino acids—methionine and cystine—and they developed negative nitrogen balances. Restoration of nitrogen equilibrium by the addition of a supplement of methionine suggested that methionine alone is capable of furnishing the entire requirement of man for sulfur amino acids.

Experimental deficiencies of histidine (93) and arginine (94) failed to induce negative nitrogen equilibrium. However a deficiency of histidine gave rise to a biochemical defect and the arginine deficiency gave rise to an anatomical lesion; these observations are discussed in detail in a later section.

Rose, Haines and Johnson (67) have determined the essentiality of amino acids in man by the use of diets composed of undisclosed mixtures of purified amino acids, starch, sucrose, centrifugated butter, inorganic salts and vitamins. The amino acid mixtures furnished 7 to 10 g. of nitrogen daily and the diet provided 2950 to 3950 kilo-calories per day. Following ingestion of this diet, the subjects came into nitrogen equilibrium within a few days. At the end of the fore-period of each subject, single amino acids were omitted from the food and the effects upon the nitrogen balance were noted. The results demonstrated that valine, methionine, threonine, leucine, isoleucine, phenylalanine, tryptophan, and lysine are necessary dietary constituents for the human. The removal of each from the experimental diet induced a pronounced negative nitrogen balance, whereas return of the missing amino acid to the diet was followed by the reestablishment of nitrogen equilibrium. It is also concluded that the remaining known amino acids in the protein molecule are not required for the maintenance of nitrogen balance in man.

Although it is quite clear that when removal of a particular amino acid induces negative N-balance, that amino acid must be regarded as essential, the converse is not true. It is not possible to conclude from the fact that nitrogen equilibrium is maintained on a particular deficiency that the missing amino acid is an unessential one. It is possible that stores of the essential factor exist which postpone for a considerable time the need for tissue destruction to supply it, which may delay the development of a negative N-balance. Another possibility is that the missing factor causes disintegration of some tissue which may be rich in it, yet this tissue represents such a small fraction of the body that its destruction is

not detectable in an N-balance study. A third circumstance in which loss of tissue to provide a needed amino acid may not be reflected in N-balances arises when there is simultaneous atrophy of one organ and hypertrophy of another, the latter process masking the negative N-balance which might otherwise occur. The tissue undergoing atrophy may supply the deficient amino acid, being richer in it than the atrophied tissue. That diet alone may cause such tissue changes is apparent from the work of Addis and coworkers (95). Thus before concluding that an amino acid deficiency which is not followed by negative N-balance is an unessential one, it is necessary to make sure that the body is not suffering anatomical, physiological or biochemical changes not reflected in the nitrogen balance.

The classification of amino acids in regard to their essentiality for man, as far as present knowledge goes, is given in Table VIII. We should like to emphasize, however, that the amino acids in the unessential column must still be regarded as only tentatively in that category, since their only claim to unessentiality lies in the fact that in very brief experiments their absence from the diet has not induced negative N-balances, a criterion which as we have shown cannot be relied upon exclusively to establish the point in question.

TABLE VIII  
*Amino Acid Requirements of Man*

Essential	Unessential (?)
Tryptophan	Cystine
Lysine	Tyrosine
Methionine	Proline
Valine	Oxyproline
Leucine	Serine
Isoleucine	Alanine
Phenylalanine	Glycine
Threonine	Glutamic Acid
Arginine (?)	Aspartic Acid
Histidine (?)	

#### V. UTILIZATION OF *d*-AMINO ACIDS BY MAN

The use of mixtures of amino acids containing considerable quantities of the racemic forms for parenteral (96, 97) and oral feeding (67) raises the question of the utilization and pharmacological action of the unnatural forms of the amino acids in man. The raising of this question is not unreasonable in view of the poor growth observed by ourselves (46) and others (46a, 46b, 46c), in rats maintained on diets containing large amounts of racemic acids and the toxic symptoms and anatomical

changes resulting from the administration of *dl*-serine to the rat (46d). The use of the racemic forms is compelled by the circumstance that certain of the amino acids: methionine, isoleucine, valine, threonine, phenylalanine, and sometimes tryptophan and lysine, can only be obtained in adequate quantities by syntheses which yield equimolar mixtures of the natural and unnatural forms. Although it is possible to resolve these products by chemical (98) or enzymic (99) methods, the available procedures are at present laborious and impractical for large scale production.

Investigations on the utilization of the optical isomers of some amino acids, carried out in the rat and the mouse (Table IX), have shown that in many respects these two species are alike in their ability to utilize some but not other *d*-amino acids. Since even these closely related

TABLE IX

*Utilization of Optical Isomers of the Amino Acids by the Rat, the Mouse, and Man*

Amino Acids	Rat		Mouse		Man	
	<i>l</i>	<i>d</i>	<i>l</i>	<i>d</i>	<i>l</i>	<i>d</i>
Methionine	+	+(100)	+	+(106)	+	+(109)
Cystine	+	— (110) (111)			+	+(112)
Phenylalanine	+	+(101)	+	+(106)	+	— (109)
Tyrosine					+	(19)
Tryptophan	+	+(102)	+	+(106) — (107)	+	— (114)
Acetyltryptophan	+(116)	— (115)			+	+(117)
Arginine	+	—? (118)			+	+(118)
Histidine	+	+(119)	+	+(120)	+	— (121)
Lysine	+	— (103)	+	— (105)	+	+(113)
Threonine	—	+(104)	—	+(106)	?	+(113)
Serine					?	+(113)
Isoleucine	+	— (131) (101)	+	— (106)		
Leucine	+	+(108) — (101)	+	— (106)		
Valine	+	— (101)	+	— (106)		

\* Excretion of 25% or less of the amino acid or its metabolites is interpreted as constituting utilization (+), while an excretion of 75% or more of the amino acid or its degradation products is equivalent to non-utilization (—).

species showed metabolic differences, the unnatural forms of tryptophan and leucine being readily utilized by the rat and scarcely at all by the mouse, it is obviously unsafe to make assumptions about man on the basis of animal studies. We have attempted to elucidate this question by



studying the excretion of the amino acids and their breakdown products in the urine after the administration of test doses of the naturally occurring and racemic forms of the amino acids. Although the deductions which can be made from the data of these experiments are necessarily limited, the procedure is of wider scope than the growth criterion technique since it permits determinations to be made with essential or unessential amino acids in a mature organism. It must be borne in mind that the results of these experiments pertain to orally, and not intravenously administered racemates.

*Tryptophan.* Although the *d* and *l* forms of tryptophan have been reported by Berg (102) and du Vigneaud, Sealock and Van Etten (115) to be equally effective in promoting growth in rats, there is evidence that mice fail to grow, or grow poorly (106, 107) on diets supplemented with *d*-tryptophan. Our studies (114) indicate that in humans the metabolism of the *d*-component of *dl*-tryptophan in the human differs markedly from that of *l*-tryptophan. The data discloses that the greater part of the metabolized *d*-component can be recovered as indigo red when the urine is treated with iodine solution within 4 to 5 hours after ingestion. Although the intermediate metabolite has not been isolated, it is clearly not utilizable by the human. This suggests that the biological value of *dl*-tryptophan is approximately one-half that of *l*-tryptophan.

*Acetyltryptophan.* These investigations (117) unexpectedly revealed that urines collected after administration of acetyl-*dl*-tryptophan, in contrast to those after *dl*-tryptophan did not yield indigo red on treatment with 0.1 *N* iodine solution. These results indicate that with the exception of a 5% urinary loss, all of the orally administered acetyl-*dl*-tryptophan may be available to man. This is in sharp contrast with the 50% utilization of *dl*-tryptophan noted above. The findings suggest the use of acetyl-*dl*-tryptophan (or better its readily soluble sodium salt) rather than *dl*-tryptophan in the reinforcement of acid hydrolyzates of proteins now being offered for supplemental alimentation. It also appears from this investigation that the metabolic deacetylation of acetyl-*d*-tryptophan results in the formation of an indole derivative which, unlike that formed in the metabolism of *d*-tryptophan, is available to man.

*Methionine.* Both forms of the amino acid induce a comparable temporary increase in the output of methionine (109). The cystine and total and inorganic S-excretion likewise failed to show significant differences. In view of our experience with tryptophan, it cannot be definitely concluded that the unnatural form of methionine is utilized, since it is possible that some abnormal metabolite of the *d*-form might have been present that was not detected. As yet, no evidence has been found which indicates the non-utilization of unnatural methionine.

*Cystine.* These experiments (112) revealed that the ingestion of *dl*-cystine caused a marked cystinuria in all subjects studied, and that the ingestion of *l*-cystine did not. However, since only 10 to 15% of the *d*-cystine is recovered in the 6 to 8 hour interval succeeding administration of the racemate, questions naturally arise as to the metabolic fate of the remaining 85 to 90% of the *d*-cystine. An additional 10% of this cystine may be lost as extra total S. Since the remainder of the *d*-cystine does not appear to yield an excess of inorganic sulfate S, methionine, or indican, it must be concluded that its metabolic fate in the human is similar to that of the *l*-fraction. Thus in man, unlike the rat, some utilization of *d*-cystine occurs. The practical implication of this interpretation of our findings is that some 25% of the unnatural cystine which may occur in acid digests of proteins would escape utilization in the human. This loss should not seriously affect the biological value of these preparations.

*Arginine.* A comparison of the output of N-metabolites, urea, amino-N, and arginine in particular, following ingestion of equimolar quantities of *l* (+)- and *dl*-arginine, suggests that the *d*- form is catabolized in the human (118). *In vitro* experiments indicate that in human liver, but not in rat liver, there occurs a mechanism whereby urea is produced from both *d* (—)- and *l* (+)-arginine. Inasmuch as our data fails to reveal any significant difference in the urinary output of amino N, it seems permissible to infer that the *d* (—)-ornithine resulting therefrom is available to the human. Moreover, since ornithine does not appear to be a constituent of proteins and since arginine isolated from proteins always appears to be of the *l* (+) configuration, it must be concluded that prior to final utilization, *d* (—)-ornithine must be converted to the *l*-isomer.

*Histidine.* Although *d* and *l* forms of histidine have been reported to be equally effective in promoting growth in mice (120) and rats (119), data obtained from excretion experiments suggest that the *d* variety is poorly utilized by guinea pigs (122), rabbits (123), and dogs (124). In our studies (121), we found that within 9 hours after administration of *dl*-histidine to adult humans a total excess of urinary histidine is excreted which is approximately equal to the amount of *d* component so ingested. This observation and the optical activity of an isolated fraction of this excess histidine suggest that *d* (+)-histidine is poorly utilized by man. This finding seems remarkable to us in view of the observations that *l* (—)-histidine is not a dietary essential for the maintenance of the N-balance in adult man. In this connection, it is also to be noted that none of the urines collected after administration of *l* (—)- or *dl*-histidine gave the atypical green color with the indican test which we reported to occur with the urines of adult human males on a histidine-deficient diet

(125). This observation suggests that the urinary substance giving rise to the atypical indican test was not derived from dextrorotary intermediates arising in the biosynthesis of histidine.

*Lysine.* Unpublished results based on changes in urinary lysine and amino N output (113) indicate that more than 80% of orally administered unnatural lysine is utilized by man. This seems remarkable in view of the observations in the rat of Weissman and Schoenheimer (126) that lysine transaminates slowly if at all and that deamination of lysine is biologically irreversible. Attention is called to the failure of administration of *d* (—)-lysine to induce the nausea or dizziness observed in the subjects maintained on a lysine-deficient diet. The availability of *d*-lysine (127) and its derivatives (128) has been studied in the rat by Neuberger.

*Phenylalanine.* The results of these experiments (109) indicate that approximately 25% of the ingested amino acid is lost by excretion within 3 hours after the ingestion of *dl*-phenylalanine, whereas only 3% or less is lost after feeding the natural form. The excretion of free and total phenols failed to show significant variations. These findings suggest strongly that the unnatural component of *dl*-phenylalanine is only partially utilized by man.

*Tyrosine.* It has been found that within 7 hours after the oral administration of *dl*-tyrosine to adult human an excess of urinary tyrosine and aliphatic organic acids are excreted which would seem to account for nearly all of the *d*-component fed. These findings are interpreted to indicate that *d*(+)-tyrosine unlike *l*(—)-tyrosine is metabolized in man in such a manner as to render it unavailable for normal physiological functions (113).

*Threonine and Serine.* Within 3 hours after the administration of the racemates of these amino acids, an increase of hydroxyamino-N, equivalent to 30 to 40% of the *l*-components of either amino acid, is found in the urine. This finding indicates a partial utilization of the unnatural forms of these hydroxyamino acids (113).

It must be apparent from these studies that until more is known of the fate and action of these unphysiological products, it would seem preferable to rely on enzymic or acid protein hydrolyzates which provide the amino acids in their natural form for supplemental alimentation. The single virtue claimed for crystalline amino acid mixtures, as opposed to protein digests—more rapid administration—may prove to be apparent rather than real, for it is possible that it is the greater loss of amino acids in the urine that allows more rapid administration. That such losses are considerable when crystalline amino acids are given, is suggested by the work of Bassett and his associates (129) who observed a rise in the

excretion of undetermined N in their experiments. A similar finding has been reported by Cox and Mueller (130) in their experiments with dogs. It would seem logical therefore to establish the merit of rapidly injected amino acid mixtures by careful studies of the excretion of amino-acid-N and other urinary metabolites.

Aside from these practical aspects, the metabolic idiosyncrasies of the mammals which are thus revealed clearly indicate the desirability of studying the unnatural amino acids and their derivatives directly in man rather than relying on inference based on animal experiments. In general, it appears that the adult human is able to utilize the unnatural forms to a greater degree than either the rat or the mouse. Apparently mechanisms for the optical conversions of the unnatural amino acids exist in man which differ qualitatively and quantitatively from those prevailing in other mammals. The findings with acetyl-*dl*-tryptophan suggest that this conversion may be effected through a biological acetylation and deacetylation mechanism which is more highly developed in man than in the lower mammals. These considerations lead us to believe that, except in instances of the formation of unphysiological products, *e.g.*, *d*-tryptophan, the utilization of the *d*-amino acids is one of degree rather than an all or none process. In other words, the availability of these substances is a function of two competing processes, (a) the rate at which the organism can convert the unnatural into the natural variety, and (b) the speed with which the respective *d*-amino acids are excreted by the kidney.

## VI. SYMPTOMS OF AMINO ACID DEFICIENCIES IN MAN

Observations on the effects of amino acid deficiencies may be divided into clinical and chemical manifestations. These two aspects of the deficiencies may or may not be directly related as cause and effect.

*Tryptophan.* In the adult, we observed that a deficient diet, continued for as long as 6 weeks, failed to produce any distinct clinical symptoms other than the loss of weight (89). The blood protein concentrations were not affected. Recent studies (132) with infants showed that within 10 days a tryptophan-deficient diet caused a marked drop in plasma proteins but not in hemoglobin which could be restored to normal levels by tryptophan supplementation within a week. Concomitant with the hypoproteinemia, the infants developed an acute anorexia which also disappeared with tryptophan reinforcement of the deficient diet. These two symptoms are analogous to those induced in the immature rat. Morphological changes observed in the rat such as a cataract and corneal vascularization were not observed in these infants, since the deficient regimen was imposed but for ten days. In the infants, as in the adults, the

excretion of tryptophan falls off sharply in the tryptophan-deficient state.

*Lysine.* A single observation in a female indicated that the lack of lysine causes partial inhibition of the menstrual cycle (90). The menstrual period was characterized by the complete absence of premenstrual distress, swelling of the breasts, headache and gain in weight, which the subject usually experienced. A subsequent experiment (133) disclosed that after the lysine-deficient diet was instituted several of our subjects noted nausea, headache, dizziness and abnormal sensitivity to noise. The symptoms which are more conspicuous in some individuals than in others cleared up when, without the knowledge of the subjects, a hydrolyzate containing lysine was substituted for the deficient hydrolyzate, and re-occurred when the deficient preparation was again taken. These episodes were associated with a rise of non-ketone organic acid output in the urine and varied in intensity with the amount of these acids excreted.

*Arginine.* Although the three subjects on an arginine-deficient diet failed to develop negative nitrogen balances within a ten day period, a study of their seminal plasma revealed such low sperm counts that the experiment was terminated promptly (94). The addition of an arginine supplement caused a prompt rise in the sperm count which became normal only after many weeks of normal diet. In contrast to our findings, Rose has reported informally that in four men maintained on arginine-free diets from 30 to 66 days, one had a low and one a high sperm count. This

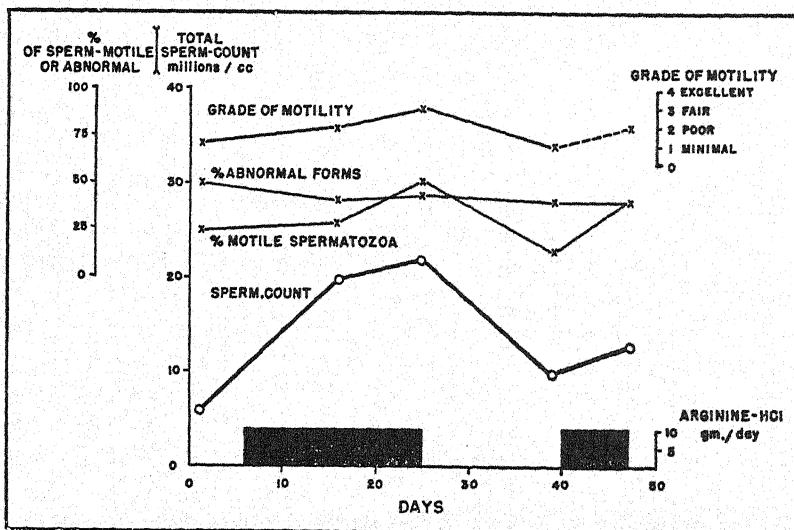


FIG. 3. The effect of arginine supplement on spermatogenesis of a patient (I. L., 28) with idiopathic hypospermia.

discrepancy indicates the necessity for further work on this problem. Our early studies pointing to a role of arginine in spermatogenesis led us to investigate the effect of arginine administration in cases of idiopathic hypospermia. To date, five such cases have been studied. It would appear that such cases are not alike in their response to this therapy. In two instances, both with very low sperm counts below 10 million per cc. virtually no response was elicited. In another, minimal improvement was noted. In the two remaining cases, definite stimulation of spermatogenesis was noted, with relapse when arginine was withdrawn, and a second increase in sperm count followed when administration of arginine was resumed. The pertinent data of these latter cases are collected graphically in Figs. 3 and 4.

The patient illustrated in Fig. 4 requires a word of explanation, for his condition was complicated by hypothyroidism, which was controlled by treatment before the onset of the present study. By a misunderstanding, his thyroid treatment was discontinued coincidentally with the commencement of the arginine therapy, and although temporary stimulation of spermatogenesis occurred, it was not maintained and the sperm count fell off as hypothyroidism became manifest. Subsequently, with the restoration of thyroid therapy, the arginine effect is clearly demonstrable, the sperm count rising when this is given and falling when it is withheld.

These observations which need to be extended suggest that metabolic relationships between dietary arginine and spermatogenesis may exist but

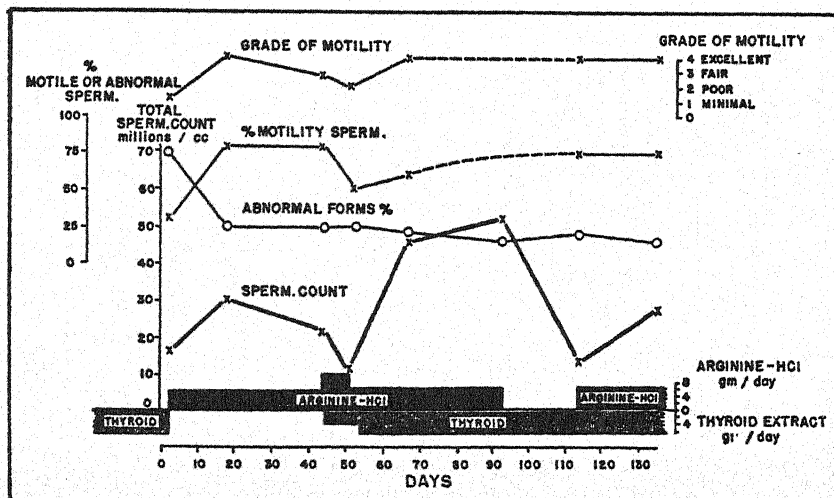


Fig. 4. The effect of arginine supplement on spermatogenesis of a patient (M. R., 30) with idiopathic hypospermia and hypothyroidism.

are subject to individual idiosyncrasies. Unquestionably a number of other factors, some of them still obscure, are concerned in the problem of hypospermia.

*Histidine.* Although 36 days of the histidine-deficient diet failed to induce a negative nitrogen balance in three male subjects, a study of the urine of these subjects showed that the diet was promptly followed by the appearance of a chromogen which gave a green color with the Sharlit indican reagent (93). The administration of histidine caused prompt disappearance of the chromogen from the urine. This abnormal metabolite, which may represent an important perversion of metabolism or one of little significance, has not yet been identified. The blood proteins were not affected by the deficiency. This is particularly significant in the case of hemoglobin, which contains some 10% histidine.

*Methionine.* In addition to the N-balance-test, we attempted to use the urinary excretion of methionine as a criterion of its deficiency, but found that the excretion continued unchanged throughout the deficiency period (134). However when methionine was restored to the diet and the negative N-balance was restored to equilibrium, the excretion rose to an abnormally high figure which indicated that in the first 3 days after the deficiency period nearly all the methionine supplement was lost in the urine. Administration of similar quantity of methionine to normal individuals has not been found to elevate the output of methionine above normal levels. This observation suggests that a transient loss of ability to metabolize to methionine had occurred during the deprivation period and that the phenomenon might be made the basis of a useful clinical test.

*Cystine.* As previously noted, the cystine poor diet failed to give conclusive N-balance results. Measurements of the cystine output during the deficiency period showed a gradual fall in the urinary cystine which rose to above normal levels on restoration of the amino acid to the diet (134). These findings might be taken to suggest that a lack of dietary cystine is not completely compensated by methionine and that some loss of tolerance to cystine was induced by the deficiency.

Our observations that in certain experimental amino acid deficiencies at least, the nature of the deficiency was reflected in the excretion of particular amino acids in the urine led us to explore the excretion pattern of amino acids in the urine in health and disease. It seemed possible that such studies might not only reveal the presence of dietary deficiencies of amino acids, but also of perversions of amino acid metabolism in pathological states. Such studies are still in their incipency and do not justify a report at the present time. The preliminary exploration of the amino acid excretion pattern under normal conditions has, however been studied



(135). Twenty-four hour urine specimens from 30 normal adult males (60 - 100 g.) on normal diets were analyzed for 12 of the known amino acids by the application of known methods and those developed in this laboratory. The daily urinary amino-N was found to fall between 200— and 700 mg., which corresponds to 2.2 to 4.5% of the total N. Calculations based on this data and the use of 136 as the average amino acid molecular weight indicate that from 1.9 to 6.7 g. of amino acids are excreted daily. The average distribution of the urinary amino acids in terms of amino acid N per cent of total amino N was found to be as follows:— arginine, 2.5; histidine, 9.4; methionine, 8.2; cystine, 1.3; tryptophan, 4.8; tyrosine, 4.7; phenylalanine, 9.4; valine, 12.0; leucine, 20.0; isoleucine, 0.0; hydroxyamino acids, 0.0; undetermined amino N, 27.7. It is noteworthy that in no instance could isoleucine or the hydroxyamino acids be detected in these urines. This finding may be due in part to the relatively low sensitivity of the tests for these particular amino acids.

#### VII. PROTEIN AND AMINO ACID REQUIREMENTS IN DISEASE\*

Although it has been customary for some years now to give generous quantities of protein in chronic febrile diseases such as typhoid fever, it is only recently that attention has been focussed upon the nitrogen losses which occur in acute surgical and medical conditions (136-141), and that attempts have been made to correct and prevent them. It has been shown that following fractures and following surgical operations in general, there occurs a marked increase in N-loss in the urine which may continue for many weeks. Similar losses occur in many acute medical conditions but with somewhat less regularity. By increasing the protein intake well beyond normal levels, or by administration of amino acids orally or parenterally, it may be possible to restore N-balance. Although striking claims have been made for clinical benefit from intensive N-therapy, the question of replacing this lost protein is a contentious one requiring further study. In spite of the apparent logic of the procedure, one must consider two factors which are difficult to evaluate: (1) the importance of loss of 'labile' or 'reserve protein' which may constitute a major fraction of the nitrogenous loss, and (2) the acceptability of the protein or amino acid supplement in the pathological state in question.

It is reasonable to believe that most of the excessive nitrogen excretion in fractures and following operations is due to the disintegration of damaged tissues. Since repair is inevitably a slow process, one may well question the need for immediate restoration of N-balance by strenuous measures.

\*See the article by Elman in the present volume, p. 269.



The so-called toxic "loss of nitrogen" in medical conditions is a more obscure phenomenon in that it is less regular in its occurrence, showing considerable variations in individual patients suffering from the same disease. In certain medical conditions destruction of tissue may play a part as is the case with surgical conditions. In others, it seems likely that the nitrogen loss represents depletion of so-called reserve protein and that the individuals who fail to exhibit the phenomenon are those who at the onset of their disease are not provided with such reserve protein. The argument for replacing or preventing loss of reserve protein is weak in view of our lack of knowledge of the nature and functions of reserve protein.

Reserve protein may be provisionally defined as a labile fraction of body tissue formed under certain dietary conditions. When a high protein diet is fed, Addis and others (95) have shown that there occurs hypertrophy of those organs particularly concerned with handling protein and its metabolic products, namely the liver and kidney. The resulting organism is well adapted to handle a high protein diet, but it is not clear that it is healthier in other respects. Lacking such knowledge, we cannot maintain the position that reserve protein should be defended or restored at all costs. Probably there is little risk in preventing labile protein loss, but there may be a definite risk in rapidly forcing protein on an organism that has lost its protein reserves, for the tissues may have lost their ability to handle it in quantity. The experiments of Holt and Kajdi (142) in rats revealed an inability to tolerate high protein diets without previous conditioning. Their observations on self selection of diets by infected rats (143) suggested a loss of tolerance to dietary protein associated with infection. Experiences with malnutrition in humans likewise indicate the need of caution in increasing food or particular foodstuffs to which tolerance may have been lost.

A possibly different type of reserve protein which should be mentioned in this connection, is that which is retained together with water and electrolyte, when carbohydrate is isocalorically substituted for fat in the diet at any level, the protein intake being unchanged. This phenomenon was first clearly demonstrated by Hellesen (144) and was subsequently studied by Cathcart and his pupils (12). The reverse process, loss of fluid, electrolyte and labile protein reserve, occurs when fat is substituted for carbohydrate. The phenomenon is clearly seen in the classical experiments of Atchley, Loeb *et al.* (145) on withdrawal of insulin from diabetic patients. From the start of insulin withdrawal, as fat combustion replaced that of carbohydrate, there occurred an increased loss of fluid, electrolyte and nitrogen in the urine.

The significance of labile nitrogen as we have said cannot be stated

at the present time. There is suggestive evidence that its presence may facilitate antibody production, hence desirable from the viewpoint of resistance against infection (146) and possibly undesirable from the point of view of allergy.

Despite the shortcomings of our knowledge in regard to the 'toxic loss of nitrogen' in acute disease, there would seem to be a number of clear cut indications for giving nitrogenous supplements. The development of hypoproteinemia either because of defective diet or disease of the kidney, liver or other organs, is *prima facie* evidence of a need for protein supplementation. Chronic infections are likely to induce a loss of more than 'reserve' protein, and attempts to combat loss by supplementary protein or amino acid therapy would seem to be indicated. Among the chronic infections tuberculosis deserves particular mention. The relation of protein depletion to the development and course of tuberculosis has been discussed by Johnston (147) and Long (148). A preliminary investigation in our laboratory has revealed that tubercular children excrete excessive amounts of tryptophan-like indole substances in the critical phases of the disease (149). Coburn and Moore (150) have recently called attention to the possible role of protein metabolism in rheumatic disease. It would seem that more attention should be paid to the possibilities of dietotherapeutic treatment of these and other chronic infectious processes for which no effective chemotherapeutic agents are yet available.

#### VIII. SPECIFIC AMINO ACID THERAPY

The thought that specific amino acids or amino acid groups may have greater therapeutic and manipulative advantages over whole proteins or protein digests in certain diseases by virtue of furnishing a ready source of some unique requirement created by the pathological processes has not been thoroughly explored and only suggestive observations along this line are now available. The efficacy of tryptophan and other amino acids in various anemias of man is still a subject of discussion (151). Oral administration of tryptophan or a tryptophan-histidine mixture has been claimed to cause rapid regression of pellagra symptoms (152). The value of methionine in preventing nitrogen loss in thermal burns has been demonstrated in the rat by Croft and Peters (153) but remains to be proven in man. Glutamic acid feeding has been claimed by Waelisch and Price (154) to benefit patients suffering from attacks of *petit mal*. We have reported some success with arginine in idiopathic hypospermia (94).

With the development of chemical methods for studying the excretion of individual amino acids and related metabolites in the urine, it seems

not unlikely that perversions of amino acid metabolism, similar to cystinuria or alkaptonuria, may be discovered where they have not hitherto been suspected.

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# The Use of Protein and Protein Hydrolyzates for Intravenous Alimentation

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## I. INTRODUCTION

The gradual development of the intravenous route as a method of administering various solutions has not only been of great practical value, but has considerable scientific interest because it has introduced a new approach to many biochemical problems. In the present discussion the clinical use of this route in the administration of protein and protein hydrolyzates will be described, based on knowledge of their metabolic and biochemical behavior. The term protein as used in this chapter will mean only the whole protein given as plasma, serum or whole blood trans-



fusions, although solutions of pure albumin are also included. The term protein hydrolyzates will be used to include mixtures of crystalline amino acids. Before discussing each of these, the possible dangers following the introduction of such substances intravenously will be discussed.

## II. DELETERIOUS EFFECTS

The clinical use of the intravenous route for the introduction of various solutions was long delayed because of the deleterious reactions following such injections. One of the commonest is the so-called pyrogenic reaction, the most frequent cause of which was discovered only as late as 1923 when Florence Seibert found that a certain bacterium grew in distilled water and produced soluble, stable substances which, on entering the blood stream, provoked severe chills and fever even if the bacteria had been killed or removed. This fundamental study was somewhat slow in being recognized so that it was really not until the last decade that pyrogen-free distilled water has become universally available, and the importance of using pyrogen-free material and apparatus appreciated. If these dangers are eliminated, deleterious effects which may follow intravenous injections are obviously due to the dissolved substance itself. Untoward reactions during whole blood or plasma transfusions have been well described by many authors and will not be further mentioned here (29, 44, 55, 73, 78).

Plasma or albumin solutions of animal origin have also been injected in the human (15, 45) either as such or following mild treatment with alkali or heat (18) to destroy their anaphylactic properties. Thus far these efforts have not proved generally successful and will not be discussed further.

The dangers from the injection of protein hydrolyzates were recognized early and were so great that this method of treatment seemed doomed to failure. The first experiments (56) were carried out in 1889. Most of these deleterious effects were described as peptone shock because it was assumed that the peptones present in the hydrolyzates were responsible. It is not clear, however, on the basis of present knowledge, whether they were due to the presence of peptones themselves, or to other materials formed during the manufacture of these early preparations. In any case, by 1913 the successful intravenous use of a protein hydrolyzate in animals was reported (38). The elimination of harmful reactions was obviously due to better methods of preparation. At the present time protein hydrolyzates have been so extensively used in humans that considerable knowledge of the untoward effects following intravenous injection has been obtained. This will be discussed under the following subheadings.

### 1. *Amino Acids and Peptides*

That amino acids themselves may be responsible for untoward reactions was the assumption of Shohl and Blackfan, who first injected a mixture of pure amino acids in the human. They found that pyrogenic reactions were just as likely to occur with a protein hydrolyzate as with a mixture of crystalline amino acids of similar composition. However, these observers offered no data to show that pyrogens were not present in the distilled water used in making up the solutions. While it does not seem physiologically sound to incriminate amino acids and peptides which are normally absorbed from the gastrointestinal tract during digestion, it was thought possible that the sudden entrance of relatively large amounts of them into the systemic circulation could provoke reactions, due perhaps to a suddenly increased specific dynamic action. At any rate, this idea has been subjected to considerable further study by injecting various mixtures of pure amino acids into animals and in humans. In these studies, however, nausea and vomiting rather than pyrogenic reactions were observed. Nausea and vomiting is a reaction which seems to be due to amino acids themselves, inasmuch as solutions containing them in pure crystalline form, when injected at too rapid a rate, are capable of provoking such an effect.

Most of the animal studies with mixtures of essential amino acids have been made on the dog by Madden and his coworkers (48), who found, however, that the best results were obtained when non-essential glycine was added, which improved the tolerance of the solution (21). On the other hand, two other non-essential amino acids were found to produce nausea and vomiting; these were glutamic acid (49) and aspartic acid (51). A contrasting experience with solutions of crystalline amino acids was reported by Cox and Mueller (13), who inferred from their observations that nausea and vomiting in dogs was much more likely due to the unnatural forms rather than to any particular amino acid.

In the human the reported observations have been few. Those of Shohl and Blackfan have already been mentioned. In another study in a surgical patient (2) a mixture of crystalline amino acids was given intravenously for 30 days without untoward effect even when the rate of injection was rapid. The present author has observed definite differences between the dog and the human with the following mixture of pure crystalline amino acids.<sup>1</sup>

<sup>1</sup> Kindly supplied by Merck and Company through the courtesy of Dr. D. F. Robertson from a formula (Vu) made up by Dr. S. C. Madden.

	g.
dl Threonine	7
dl Valine	11
dl Leucine	18
dl Isoleucine	12
l Lysine, HCL	12
l Tryptophan	4
dl Phenylalanine	12
dl Methionine	6
l Histidine	4
l Arginine	7
g. Glycine	7
	<hr/> 100

The mixture was made up 75 g. per liter, *i.e.*, 7½%, which contained 0.9 g. % of nitrogen. Glucose 5% was added before administration. When injected in dogs considerable nausea and vomiting followed the injection of this mixture as compared, for example, with a solution of hydrolyzed protein (Amigen<sup>2</sup>). In humans the results were just the reverse, *i.e.*, the pure amino acids were tolerated better, as shown by the fact that the solution could be injected far more rapidly without provoking nausea and vomiting, as compared with a solution of hydrolyzed protein (Amigen). Moreover, in three trials with a solution of pure glutamic acid alone (one liter contained 30 g.), no reaction was observed following rapid intravenous injection in convalescent patients.

## 2. Other Substances

Undoubtedly present in hydrolyzates but not in pure mixtures of amino acids are other substances which may be responsible for some of the untoward reactions. This was undoubtedly true of many of the earlier preparations of protein hydrolyzates which seemed to provoke a higher incidence of untoward effects as compared with present preparations, which are presumably purer. A specific study was made by Hopps and Campbell (41), who showed by *in vitro* tests with guinea pig muscle that enzymic as well as acid hydrolyzates of casein contain substances assumed to be either like histamine, peptones or tyramine. Their presence would seem to explain such clinical phenomena as flushing of the skin, various vasomotor and other signs and symptoms whenever the injection rate is too rapid. Further observations are needed in order to study the effect of, and eliminate substances of this nature as much as possible.

<sup>2</sup> An enzymic digest of casein and pork pancreas, manufactured by Mead Johnson and Company, Evansville, Indiana.

### 3. *Products of Bacterial Contamination*

Bacteria and/or the products of bacterial growth may readily be responsible for severe pyrogenic and other reactions, which may even be fatal. Such substances may readily form, particularly because solutions of hydrolyzed protein are excellent culture mediums. It is probable that contamination may result in such rapid bacterial growth that reactions may occur within a relatively few hours after the sterile solution is opened to the air. Suppose that a few bacteria gain entrance and begin to multiply immediately. Eventually, of course, the solution becomes turbid and the evidences of contamination are readily apparent upon inspection. There is, however, a period before the solution becomes turbid in which bacterial growth has been considerable. The injection of such a clear solution is obviously fraught with danger, yet it may readily be avoided by the simple precaution of injecting the clear solution as soon as the sealed container is opened. One liter of such a solution can be given to an average sized adult within two or three hours. It is unlikely that sufficient bacterial growth could take place within this period to produce an untoward reaction. On the other hand, if such a flask is opened and 6 to 8 or more hours allowed to elapse before the injection is started, such growth could occur. It may be that a few untoward and perhaps fatal reactions may have been caused by such a circumstance. The obvious lesson is the avoidance of contamination by (a) the use of closed systems similar to those recommended for all intravenous injections, and (b) starting the injection immediately after the container is opened, and completing it within a period of 3 or 4 hours at the most.

### 4. *Allergens*

Solutions of hydrolyzed protein may contain substances to which a particular patient is sensitive. This undoubtedly occurs even though complete and thorough tests show that no anaphylactic substances are present in the product. Allergens comprise a variety of things and are the obvious cause of urticaria following an intravenous injection. Other manifestations of allergy are severe itching of the skin, patchy edema often called angioneurotic edema, and skin rashes. Each of these has been observed occasionally. The author once saw a pronounced edema of the mesentery at operation in a patient who, within the previous 24 hours, had received an injection of hydrolyzed protein without apparent reaction at the time. Serious allergic reactions of this sort have been rare, although it is possible that a fatal result may occur. The incidence is not known, but must naturally be compared with the fatal

reactions which have been observed following blood and plasma transfusions, which even in recent years have been reported as occurring about once in every one to three thousand transfusions (44).

Actual experiences with the rate of reactions following the intravenous injection of solutions of hydrolyzed protein have been published (28), but most other reports deal with single reactions or the analysis of a relatively small number of cases. The present author has carefully studied each consecutive injection during a period of 9 months, the results of which are presented herewith:

Total number of patients receiving Amigen	352
Average injection per patient (each 1 liter)	8
Largest number of injections in one patient	53
Total number of injections (each 1 liter)	2,729
Total number of untoward reactions	22(0.8%)
Nature of Reactions:	
Pyrogenic (chill with or without fever or fever without chill)	11(50%)
Allergic (itching, urticaria, rash, edema)	7(31%)
Miscellaneous	4(19%)

The four miscellaneous reactions were described as disturbing subjective sensations as follows: tingling and suffocating, vomiting, faintness and perspiration, headache and dizziness, all of which seemed severe enough to discontinue the injection. Of the other 18 reactions, 12 were observed in patients who received Amigen previously and subsequently without incident. Of the total of 22 reactions in only 10 was the injection a first one, and did the reaction constitute a contraindication. In many of these no reactions might have been observed had the injection been repeated. In two of these, reactions occurred with subsequent injections of other fluids. There were no deaths attributable to the injections. The speed of the injection was somewhat slower than that of other intravenous infusions. In general, two hours was the average duration for one liter. With faster rates nausea and vomiting were encountered occasionally, and this was not recorded as a reaction unless these symptoms persisted and the infusion was therefore discontinued. In all others the symptoms disappeared as the flow was slowed down.

### 5. Other Deleterious Effects

Regarding phlebitis and thrombosis, the influence of hypertonic solutions is well known. The influence of pH seems to be particularly important with solutions of amino acids because of their great buffering properties. Actual experimental study has shown that phlebitis is more likely following injections of solutions of amino acids with an acid reaction (42). Another possible harmful effect of injecting a solution

of amino acids at a low pH is the fact that they are capable of significantly lowering the carbon dioxide combining power of the blood. For example, solutions of hydrolyzed protein at a pH of 5.5 may significantly reduce the CO<sub>2</sub> combining power of the blood (12), whereas when neutralized to a pH of 6.5 they have but slight effect on the CO<sub>2</sub> combining power and none on the pH of the blood, as shown by actual measurements in 2 patients (42). After the injection during 8 hours of 3 liters of a 5% solution, there was a drop of but 6 and 8 volumes per cent respectively. This is another reason why amino acid solutions should not be given at a pH which is very far from the neutral point.

### III. THE USE OF PLASMA TRANSFUSIONS

Plasma transfusions have been extensively used in the past decade as a method of combating certain types of surgical shock, especially that due to acute loss of plasma protein. In a somewhat small number of cases, solutions of pure human albumin have been employed for a similar purpose. Whole blood is also a method of introducing protein intravenously and includes the intracellular protein hemoglobin. The use of plasma and whole blood for meeting acute needs is fairly well understood, and will not be discussed in great detail. For such purposes they may be looked upon as methods for the immediate replacement of acute losses due to one cause or another (22). If the loss is replaced soon enough, clinical response is satisfactory and if the loss does not persist, such replacement restores the deficit without further treatment. Common clinical conditions in which plasma transfusions have been used are extensive burns, intestinal obstruction, general peritonitis and pneumonia, all of them conditions in which extravasation from the blood stream into the tissues has occurred, the fluid consisting essentially of plasma. These conditions, however, shade imperceptibly into other situations in which red blood cells have also been lost. These include various types of trauma, both accidental and operative. In the latter conditions whole blood rather than plasma is indicated in order to correct the deficit in red cells as well as in plasma protein. In either case the indications are rather clear, and the results usually effective unless too long a period has elapsed between the loss and its replacement. Such a delay permits irreversible changes to develop which, if severe, cannot be overcome by any methods known at the present time. Since it is expected that the detailed consideration of these problems will be taken up in a later volume of this series, no further discussion of these matters will be given here.

Plasma transfusions as a method of supplying protein nourishment present quite a different problem from that of correcting the acute

protein deficits just mentioned. In contrast to its obvious mechanism in such conditions, the injection of whole protein directly into the blood stream as food introduces a new physiological problem. Protein nutrition normally begins with the absorption of amino acids from digested protein in the gastrointestinal tract. Amino acids, therefore, are the normal starting substances in the blood and body tissues. To understand how whole protein is utilized when it enters the blood stream directly requires new metabolic information. What is known will be briefly discussed.

There are at least two theories regarding the metabolic changes in plasma protein introduced as a transfusion. One is the traditional view based upon the behavior of food protein which, as is well known, is reduced to amino acids before absorption and then resynthesized to body protein. According to this mechanism, plasma protein injected into the blood stream must similarly be degraded to its constituent amino acids by means of cellular or circulating enzymes and then resynthesized to tissue or other protein. This would mean, of course, some loss of nitrogen inasmuch as the amino acid composition of most tissue protein is unlike plasma protein. Moreover, it presupposes that plasma protein contains all the essential amino acids required to make tissue protein. This is true of some of the plasma proteins, but not all of them (5, 10, 37). The second theory has been propounded by Whipple and his coworkers (47, 80, 81). According to evidence presented by these observers, plasma protein may replace tissue protein without loss of nitrogen, *i.e.*, that it may be used without being broken down to its constituent amino acids. This theory is supported by recent studies (64) indicating that many metabolic interchanges may alter the protein molecule without hydrolysis to amino acids or peptides.

Actual observations on the metabolic changes following plasma, serum, or whole blood transfusions have been fairly extensive. Studies of nitrogen balance date back to 1875 and have been reviewed in detail elsewhere (19). In brief, these observations have shown that nitrogen balance can be achieved during the injection of plasma protein although there was some evidence that (a) incompatibility even when overt reactions did not occur could lead to increases in urinary excretion of nitrogen, and (b) delayed loss of nitrogen could occur some days after the termination of the injections. However, these studies have thrown insufficient light on the question of what actually happens to the injected protein, which is now discussed under the next heading.

### 1. *Fate of Injected Plasma Protein*

Many observers have noted that intravenously injected plasma protein leaves the circulation fairly rapidly, although not nearly as rapidly



as glucose or amino acids. This is not surprising in the case of large and long-continued injections. For example, in one of the earlier observations (39) canine plasma was injected intravenously in dogs as the sole source of nitrogen. It was possible to calculate on the basis of plasma volume determinations that only 3.6 to 15% of the total amount of the injected protein remained in the circulation at the end of two weeks. Inasmuch as there was no increase in urinary nitrogen it was obvious that the rest of the protein escaped from the circulation and must have been retained in the tissues in some form. In a somewhat similar study on protein-depleted dogs (20) plasma transfusions were given each day for a week. At the end of this period only 10% of the injected plasma protein could be found in the blood stream. The nutritional hypoproteinemia which was completely corrected and indeed overcorrected by this time did not persist, so that within two weeks, the level of albumin had dropped again to its previous level. Moreover, during this period much of the nitrogen retained during the week of treatment appeared in the urine. These observations are not surprising in view of the large amount of protein injected in relation to the relatively small amount of normally circulating plasma protein.

Similar observations were made with single injections. For example, in two similar studies (40, 54) in dogs, careful estimations of plasma volume and protein concentration indicated that the entire amount of injected protein left the circulation in 3 to 8 hours. In another study (32) dogs were given plasma protein containing radioactive lysine which permitted an accurate study of the fate of the injected protein. It was found that within 24 hours 50% of the injected protein had disappeared from the blood stream and by 6 days 75% had gone. This was true not only of normal dogs, but also of dogs in whom surgical shock had been produced by intestinal and muscle trauma. In other experiments (3) on animals, the behavior of plasma protein was investigated and evidence obtained of a rapid disappearance and appearance of plasma protein under a variety of experimental conditions.

In the human, several studies have been made of the effect of single injections. In one study by Hayward and Jordan plasma transfusions of 800 to 1200 cc. were given 19 patients, most of them presumably normal. As shown by changes in the concentration of hemoglobin, there was an immediate increase in plasma volume, which returned to normal in 2 to 5 days. However, the plasma protein concentration in general remained unchanged, showing that the injected protein left the circulation along with the injected fluid. This was especially striking in two patients with nutritional edema and low serum protein levels of 4.5 and 4.7 g. per cent. Neither value was affected by the plasma transfusion,



nor was there much evidence from hematocrit readings that even the fluid itself was retained. Thus in one of these patients 6 hours after the transfusion, there was no evidence of hemodilution, whereas in the other there was only slight hemodilution which had disappeared some time between the injection and 24 hours later. The authors conclude that the injected plasma protein rapidly disappeared from the circulation. The observations in these two cases can be duplicated by many clinicians who have successfully tried plasma transfusions for the correction of nutritional hypoproteinemia. Similar single injections of 700 to 2100 cc. of plasma were given by Sharpey-Schafer and Wallace. Thirty-one observations in nearly as many individuals were described. Both normal and concentrated serum were used but the findings were the same with each and in general showed a transient hemodilution without change in protein concentration. This indicated that the injected fluid and protein stayed in the circulation only a short time. Moreover, there were several cases in which there was no hemodilution with no change in the serum protein concentration, indicating an almost immediate disappearance of the injected fluid, including protein. Contrasting findings were obtained in several patients who were given serum immediately after a venesection. Hemodilution persisted in these cases, which suggested that the injected fluid did not leave the circulation; presumably this was also true of the protein, although no serum protein studies in these cases were reported.

Similar observations regarding the prompt disappearance of injected protein were made by Janeway and his coworkers, who utilized concentrated solutions of pure human albumin. They found that, after single injections, albumin leaves the blood fairly rapidly both in normal humans as well as in those with pronounced blood loss, except that the rate is much slower in the latter group. The same workers studied the effect of repeated injections of human albumin in cirrhosis of the liver which showed that a large proportion of the injected protein leaves the circulation.

A detailed metabolic study of what actually happens to the protein in plasma transfusions after it leaves the circulation has been carried out by Fuller Albright and his coworkers in a patient with idiopathic hypoproteinemia. By carefully measuring nitrogen as well as potassium and phosphorus values, evidence was obtained which indicated that roughly 50% of the protein so injected was retained by the body and remade into tissue protoplasm, the other half appearing in the urine as urea and ammonia. This study will undoubtedly be carried out in normal individuals as well as in those suffering from uncomplicated protein starvation, and the results will, of course, be of extreme value in answering

the question of the nutritional importance of plasma transfusions as a source of protein food. The studies of Albright and his coworkers, moreover, have already shown that the production of protoplasm from plasma protein as well as the excretion of nitrogen is not immediate, but requires a number of days, presumably about 6 to 10, after the transfusion.

These findings fit in with the present dynamic view of protein metabolism; in accordance with this concept, plasma protein is undoubtedly being made and destroyed constantly. Study with isotopic nitrogen containing amino acids by Schoenheimer and his coworkers (65) has indicated that the half life of the serum proteins in the rat is about 14 days. In other words, about half of the circulating protein undergoes chemical changes during a period of 14 days and is replaced in the normal organism by new molecules. It may be assumed, therefore, that injected plasma protein will undergo similar changes. One could expect it to remain in the circulation only when it is used to replace an acute deficit produced, for example by hemorrhage. On the other hand, the situation is basically different when the deficit is nutritional in origin. The most significant difference is the fact that the fall in plasma albumin is associated with a depletion of the protein tissue throughout the body. The quantitative relation between these two has been estimated to be 1 to 30 respectively (63, 76). It is easy to understand, therefore, why one cannot correct the plasma protein deficit alone by merely injecting a large amount of plasma into the blood stream. Because the deficiency involves the whole body, the compartment of protein in the circulating blood cannot be viewed as an isolated case.

## *2. Intravenous versus Oral Administration of Plasma Protein*

In considering plasma as protein food its metabolism when injected intravenously must necessarily be compared with the behavior of the same protein when taken by mouth, inasmuch as there is such a difference in the mechanisms involved. Study of the nutritional value of plasma protein when given by mouth has resulted in a variety of results. For example, it has been shown (52) that following oral administration positive balance can be achieved in dogs with precipitated washed bovine serum at a level comparable to that of casein. This same worker (53) as well as others (77, 80) has shown that beef serum by mouth is of high nutritional value in respect to plasma protein regeneration in protein depleted dogs. On the other hand, growth experiments with human plasma have definitely shown nutritional deficiencies (10, 37) which are confined to the albumin fraction. By contrast, globulin and fibrin are of high nutritive value. The deficiency in albumin apparently is due to a lack of isoleucine and tryptophan; this was shown not only

in feeding experiments, but in actual chemical analysis (5). A decisive experiment showing the difference between the metabolism of dog plasma by mouth and by vein was carried out by Holman, Mahoney and Whipple. It was observed that oral administration failed to correct hypoproteinemia and led to an increased loss of urinary nitrogen, in contrast to parallel experiments in which the same amount of plasma was injected. These observers concluded "evidently the protein by vein is a little more completely utilized to form new protein in the body than the same protein given by mouth."

Because plasma contains several different proteins, each in varying proportions, each possessing different nutritive values, it would seem obvious that varying results will follow the use of different specimens of plasma. On the other hand, it seems clear that the metabolism of plasma protein when injected into the blood stream is physiologically unique and requires much more study before it can be evaluated as a sole method for parenteral protein alimentation.

### 3. *Plasma as Parenteral Protein Food*

In actual clinical practice plasma and whole blood transfusions have been fairly extensively used in malnourished patients in attempts to correct protein deficiencies. A lasting correction of anemia will follow the injection of enough red cells but the effect on chronic hypoproteinemia even with large amounts of plasma protein has been disappointing, as compared with the good results in acute hypoproteinemia. The reasons are probably those already discussed. Nevertheless, it must not be inferred from these considerations that plasma transfusions have no value in the treatment of chronic protein deficiency. While it is true that appropriate amino acid mixtures represent a more convenient, economical and physiological method of intravenous protein feeding, the use of plasma and whole blood are important adjuncts to this therapy. Even when the protein level of the blood is unaffected, an occasional plasma or whole blood transfusion, especially when used as a part of a program of full parenteral alimentation, has a beneficial influence as judged by the improved clinical behavior of the patient.

## IV. PROTEIN HYDROLYZATES

At least two types of protein hydrolyzates have been extensively studied, only one of which will be discussed in detail. The first is represented by gelatin, which is in reality a mild hydrolyzate of connective tissue protein, although it is often considered to be a solution of whole protein. Connective tissue protein is obtained in solution by boiling the tissue, usually bone or skin, and extracting it in this way.

Gelatin is unique among proteins in that it becomes hydrolyzed under these conditions, requiring neither acid, alkali or enzymes. The solution as finally obtained, therefore, while largely one of whole protein, also contains a variety of smaller molecules and indeed in some preparations even single amino acids. In recent years gelatin solutions have enjoyed a renaissance in the treatment of shock (30, 57), having first been used for this purpose during World War I. As a plasma substitute its effectiveness is due to the colloidal osmotic pressure exerted by the preponderance of large molecules present in the solution. However, much of the material, presumably the smaller molecules, appears rapidly in the urine. Little is known of the metabolic behavior of gelatin, and there is some dispute as to whether it acts as a foreign body, or is subjected to various anabolic changes (7, 40, 58, 60).

The other protein hydrolyzate is one in which the digestion has been carried largely to the stage of amino acids; these may be compared with pure crystalline amino acids themselves. Of the two, protein hydrolyzates have been extensively employed as a means of supplying protein food parenterally. Because pure crystalline amino acids are expensive, they have had relatively limited clinical use and most of the discussion, therefore, will be confined to the effect of solutions of hydrolyzed protein containing complete mixtures of amino acids.

### 1. *Metabolism of Amino Acids Given Intravenously*

The introduction of amino acids directly into the blood stream, unlike the injection of whole protein as a plasma transfusion, presents no unusual physiological problem inasmuch as normal protein nutrition involves the entrance of these same building stones of protein into the blood stream. There is, however, a difference between an injection into the systemic veins, whereby all the amino acids as such quickly reach all the tissue at one time, and the gradual, and perhaps inconstant absorption of variable amounts of various amino acids, after being subjected to bacterial action, into the portal vein and thence to the liver alone during the digestion of food protein. In order to evaluate the metabolism of intravenously injected amino acids, this difference must be scrutinized.

*a. Differences between Oral and Parenteral Administration.* The utilization of amino acids injected into the systemic circulation as compared with their absorption into the portal circulation theoretically may differ in at least three ways. *First* there is the influence of the liver. It might be assumed that the introduction into the peripheral vein would be advantageous in that deamination by the liver would be less extensive and that amino acids would reach tissues more rapidly and directly and

thus permit more efficient utilization. Amino acids which must pass the liver first and reach the other tissues second might suffer considerable loss of nitrogen. On the other hand, the role of the liver in preparing amino acids for utilization must be important inasmuch as some synthesis occurs as well as deamination. The *second* difference between the intravenous and the oral route is the fact that amino acids injected into the blood necessarily all reach the tissues at the same time. This would seem also to increase the likelihood of efficient utilization because of the synchronous presence in the blood stream of all of the essential ones. This factor is inevitable during intravenous injection, but might not be so true when amino acids are absorbed from the gastrointestinal tract, particularly when digestion is somewhat impaired. In an experimental study (28) with an incomplete mixture of amino acids the importance of this time relationship was shown by a failure of nitrogen balance when tryptophan was injected even 6 hours after the injection of an hydrolyzate containing all but this amino acid. This observation may explain the fact that intravenous injections of amino acids produce responses in the nitrogen output immediately, unlike the delay usually observed when protein is given by mouth. The steplike increase or decrease in nitrogen output following the addition or withdrawal of protein by mouth is not seen with the intravenous injection of hydrolyzed protein. This delayed effect of protein when given by mouth may be due to a lag in digestion and absorption and thus would indicate that the intravenous route is more efficient, presumably because all of the essential amino acids reach the tissues at the same time. A *third* difference is based on the possible influence of bacterial action in the intestines on the amino acids before they are absorbed. Little is known of such action, but obviously parenterally injected solutions are not subjected to such influences.

Actual observations, for the most part, have thus far shown very little difference in the utilization of amino acids, whether they are injected into peripheral veins or absorbed from the gastrointestinal tract. For example, Henriques and Anderson, in a 16-day experiment with full intravenous alimentation, showed that nitrogen balance was the same during a 10 day period while a solution of hydrolyzed protein was injected into the systemic veins as it was during the subsequent 6 days while the injection was made directly into the splenic vein. Confirmatory of these findings is a more recent study (27) which showed the same degree of nitrogen retention and serum protein regeneration with an amino acid mixture given by mouth and by vein. Clinical studies in infants (70) have shown just as good nitrogen balance with intravenous injections of amino acids as with the same amount of food protein by

mouth. This was also shown to be true of older children (35). In adults after operation (9) evidence was also obtained that there was just as good nitrogen balance during periods of complete intravenous alimentation immediately after operation as during subsequent periods when food was taken by mouth even though the conditions in the former periods favored a greater loss of nitrogen. An analogy might finally be drawn from many observations with intravenous glucose in support of the idea that the systemic introduction of nutriment is just as effective as its absorption into the portal stream. A contrasting experience has been reported (51) in which a mixture of pure crystalline amino acids was found to be better utilized when given by mouth than by intravenous injection.

*b. Fate of Amino Acids After Intravenous Injection.* This was first studied in 1913 by Van Slyke and Meyer. Although part of a larger investigation into the fate of protein digestion products in the body, this study marks a milestone in our knowledge of the behavior of amino acids after intravenous injection. A few of the findings will be described. The amino acid mixture was obtained by hydrolysis of casein or beef and was injected into anesthetized dogs in fairly large doses (1.5 g. per kg.) over a period of one-half hour, and the animal killed from one-half to several hours after the end of the injection. Even with this large dose and at this rapid rate of injection, but 5% of the injected amino acids was left in the blood one-half hour after injection, and at the most 11% excreted in the urine. All tissues showed increases in amino acid content. The liver showed the greatest increase, yet returned to normal the quickest. At a time when the muscle content was still high, the liver had given up most of its amino acids. This finding shows the importance of the liver in the metabolism of amino acids even when introduced into the systemic circulation.

Other experiments have also shown that amino acids disappear rapidly from the blood stream after intravenous injection without significant excretion of amino acids in the urine (23). This must indicate a tremendous avidity for these building stones of protein by all of the tissues. The eventual behavior of amino acids after they leave the blood stream is not known in detail, although it would seem that it probably differs very little basically from the metabolism of amino acids absorbed into the portal circulation. In other words, some are metabolized into non-protein nitrogenous substances such as creatine, purine, etc.; others are deaminized, the nitrogen converted to urea and the rest utilized as carbohydrate or to form other amino acids, the remainder are synthesized to form tissue or other protein, hormones, etc. That relatively little is lost as urea and that protein synthesis actually occurs is shown

by the fact that the injection of appropriate amino acid mixtures leads to the achievement of positive nitrogen balance (8, 24, 34, 49, 68) and to increases in serum albumin (11, 14, 24, 49) even when no other form of protein nourishment is given. Under conditions of tissue damage and severe infections in well nourished individuals, evidence has been obtained that amino acids are not utilized but are deaminized and excreted as urea. However, this unusual behavior is also true of food protein taken by mouth and therefore will not be discussed further.

*c. Other Physiological Considerations.* Because of their metabolic interdependence, it is obvious that no one of the various nutriments can be considered alone. In the case of amino acids these relationships are of special interest and are therefore discussed in detail.

*Water and electrolyte* have an obvious relationship to the metabolism of amino acids. Although whole protein requires more water for its metabolism than glucose, amino acids are probably different and may act more like glucose in that they give up water during the process of synthesis. On the other hand, water is required for the excretion of urea resulting from deaminization. An even more important consideration is the fact that tissue protein in the form of protoplasm contains 4 or 5 g. of water for every gram of protein. For this reason large amounts of water undoubtedly are necessary for the synthesis of cell protoplasm. In regard to electrolyte, the synthesis of cell protoplasm requires both potassium and magnesium rather than sodium salts. Amino acids which are manufactured into protoplasm will obviously need such salts. Very little, however, is actually known of the amount required and whether there is a sufficient store of them in the body in depleted patients. Further studies will undoubtedly answer many of these questions regarding water and electrolyte relations.

The relationship between the metabolism of intravenously injected amino acids and *glucose* is of both theoretical and practical interest. Whether the utilization of amino acids requires the simultaneous presence of glucose has been answered on theoretical grounds in the affirmative, although a full answer awaits further investigation. If glucose is necessary, how much is required? The evidence now available is meager and somewhat conflicting. For example, Farr (31), in children, observed no difference in the utilization of intravenous amino acids whether glucose was injected at the same time or not, although the injections were supplementary ones and formed but a part of the total dietary intake. Drury and Greely, in rabbits, found that the glucose requirement was greatly reduced by the simultaneous administration of amino acids. This was not due to the deaminization of the amino acids but to some other specific factor which was present even when the oral route



was used. Brunschwig and his coworkers (8), on the other hand, presented evidence in humans showing that the injection of amino acids without glucose tends to lead to a greater output of nitrogen in the urine than when glucose is added. However, nitrogen balance has been obtained by the present author during total intravenous feeding without a full caloric intake. Under such conditions the patient's tissue fat undoubtedly supplies the added calories. Experimental evidence in support of this idea has been reported elsewhere (21). The matter requires further study.

*Vitamins* must have an important relationship to the metabolism of amino acids, although very little is known thereof. Suggestive observations have been made in regard to the relationship of riboflavin to protein storage, but no specific data have been published. The various enzymes which control the synthesis of protein might be mentioned along with vitamins because such enzymes obviously govern the metabolism of protein and amino acids. Of more practical significance is the fact that certain of them can direct the synthesis of amino acids into specific channels. For example, it would be interesting to speculate on the possibility of isolating an enzyme which has the property of directing the synthesis of amino acids into plasma protein rather than in other types of protein. The practical advantages of such a procedure are obvious.

Control of hunger by intravenous injection of amino acids poses an interesting question which is difficult to answer in view of the fact that any intravenous injection evokes an unpleasant reaction on the part of most patients. This would tend in itself to abolish any sense of hunger and to promote anorexia instead. Nevertheless, many isolated observations indicate that the intravenous injection of amino acids may satisfy the sense of hunger almost as much as the ingestion of the same amount of protein nourishment by mouth.

The effect of intravenously injected amino acids on gastrointestinal physiology has been studied briefly, and in human beings found to decrease tonus and produce a disappearance of contractions (79). In dogs, the same worker found that the injection produced an increase in duodenal motility (59). The effects of rapid intravenous injections of amino acids on the production of nausea and vomiting in dogs and humans have already been discussed.

Much remains unknown of the details of the metabolism of amino acid mixtures after intravenous administration, particularly as to the relative degree to which they are subject to anabolic or catabolic processes. Studies of respiratory quotients and D/N ratios have not been carried out and would undoubtedly add to our knowledge. Moreover, little actually is known of what type of mixture is best suited for various purposes.



To be sure, W. C. Rose (61) has meticulously studied the influence of variations in the proportion of the essential amino acids when given by mouth to growing rats and has formulated an "ideal" mixture, which answers the question regarding growth in specific terms for the rat. He has also studied the problem in adult human beings and has found (62) a different formula for the most efficient maintenance of nitrogen balance by oral ingestion. On the other hand, the most efficient mixture for rebuilding tissue protein is not known, nor has the influence been studied of added potassium and magnesium, which are always present in cell protoplasm. Madden and his coworkers (48-51) have tried various intravenous mixtures of pure amino acids on dogs in order to determine which formula is the most efficient for the regeneration of plasma protein after plasmapheresis, as well as for the maintenance of nitrogen balance. Much important information has been obtained indicating that what is needed for the latter may not be so for the former. The problem is obviously a difficult one for each of the 10 essential amino acids. Eventually, however, the data probably will be adequate and an "ideal" mixture for intravenous use in the human being may be formulated both for protein loss directly as well as depletion from starvation. If crystalline amino acids in their natural forms are then generally cheap and available, the question of the most efficient mixture can be answered to perfection, or a hydrolyzate can be fortified so as to closely approach this ideal. Until then it is necessary to use hydrolyzates which are known to contain a sufficient proportion of the essential amino acids. While a little wasteful, such a mixture is no more so than whole protein which we normally eat and assimilate, a large part of which is not needed and is excreted in the urine as nitrogen.

## 2. Degree of Digestion in Protein Hydrolyzates

The use of protein hydrolyzates instead of mixtures of pure amino acids leads to the interesting and unanswered question of what is the most appropriate degree of digestion? With acid hydrolysis complete digestion is relatively easy and a product can be made which contains amino acids only with no peptides. Enzymic hydrolysis, on the other hand, is difficult to carry to completion and as a result pure amino acids form but 50 to 70% of the mixture. The rest of the protein is incompletely digested and remains in the form of peptides. From the theoretical point of view the existence of peptides may be advantageous inasmuch as it saves one step in the long process of synthesis. If this is true, the question may be asked—how large may the aggregates of amino acids be and still be used to form tissue and plasma protein? Bergmann (4) has offered evidence that small groups of amino acids may act as basic

elements which may perhaps be used to build up a variety of larger protein molecules. Fischer (33) has reviewed the work on utilization of amino acids and peptides by tissue culture and has stated that large peptides can apparently be better utilized by cells in certain tissue cultures than small peptides or single amino acids.

A number of suggestive clinical observations have been made with various hydrolyzates in the treatment of starved patients unable to take anything by mouth. The findings have thrown some light on the utility of various types of protein hydrolyzates. For example, after the Bengal famine in India a protein hydrolyzate solution containing a large proportion of peptides, many presumably of large size, was injected intravenously in the treatment of many of the moribund cases. Krishnan and his coworkers (46), who carried out this study, were able in this way to reduce the previously observed high mortality in these individuals. Their preparation was called a peptone-glucose solution and was made by the digestion of pork with papain. By contrast, solutions of glucose alone, or indeed, plasma or whole blood transfusions, were relatively ineffective. After the end of the European phase of World War II a number of the extremely emaciated victims of German concentration and prison camps were treated with injections of both plasma and protein hydrolyzates. The details of these observations are not available, but from briefly published data (75) an acid hydrolyzate proved much less effective than an enzymic hydrolyzate. It is probable that the former contained only amino acids, whereas the latter contained many peptides, but not as many as the preparation used in India. Unlike the observations made by Krishnan, Edge (17) found plasma fairly effective in the treatment of 300 Englishmen suffering from the effects of prolonged starvation while German prisoners of war; he did not use hydrolyzates. On the other hand, Stannus (71) cited two starved patients in whom glucose and saline plus liver extract were effective after blood and plasma transfusions had failed. Another possible factor in the striking results described by Krishnan and his coworkers may be the presence of potassium and magnesium in their hydrolyzates, which was prepared from cellular protein (pork), unlike the other preparations which were made from pure extracellular protein (casein) and therefore contained neither of these salts in significant amounts. Further studies are obviously needed.

Hydrolyzed protein can also be prepared so that the particles are of sufficiently large size to exert colloidal osmotic pressure. Such preparations may act as plasma substitutes. This is really what happens when gelatin solutions are used, as already mentioned. A papain digest of casein has been described by Brinkman (6), which seems to have col-

loidal as well as nutritional attributes when used in humans. Further studies along this line are awaited with considerable interest.

## V. THE THERAPEUTIC USE OF AMINO ACIDS

### 1. *Contraindications*

First of all, the possible contraindications to the intravenous injection of amino acid solutions must be mentioned. It is obvious, of course, that any turbidity or precipitate in the solution constitutes an absolute contraindication. In other words, only crystal clear solution should be injected. This is especially important because of the fact that plasma is ordinarily opalescent or even turbid and yet is frequently given without danger. It is obvious that turbidity in a solution of amino acids must be assumed to be due to bacterial contamination unless proved otherwise. The intravenous injection of amino acids should always be discontinued in the presence of any reaction even though it is a simple pyrogenic reaction. These have been discussed under another heading. Ordinarily this should not be counted as a contraindication to further injections. However, when the reaction is one of allergic sensitivity, *i.e.*, if the patient exhibits urticaria, severe itching, skin rash or angioneurotic edema, further injections probably should not be given.

The only other contraindications to the intravenous injection of amino acids that have been expressed deal with the presence of severe hepatic or renal disease. In the case of the former, Hopps and Campbell (41) have described a case in which at autopsy severe hepatic damage was observed. Contrasting evidence has been presented by Stewart and Rourke (72), who injected solutions of hydrolyzed protein in several patients with severe hepatic disease without deleterious effect. It is true, of course, that the blood level of amino acids is high in severe hepatic insufficiency and that this may be considered as a contraindication, although further observations are obviously necessary.

The presence of severe renal impairment is sometimes used as a contraindication to the administration of any protein food inasmuch as such food leads to the production of additional ammonia and urea, which imposes an excretory task upon the kidney. This can be minimized by administering pure glucose and water as the only nutritional substances, thus reducing the excretion of nitrogen (and salts) to a minimum. These considerations apply equally to the injection of amino acids and may have an application in surgical patients whenever renal impairment follows a serious operation. When a surgeon is confronted by anuria during the postoperative course, all intravenous therapy should be limited to glucose in water in order to spare the kidney, aside, of course,

from the indications for plasma, whole blood or basic solutions to combat acidosis.

### 2. *Correction of Acute Protein Deficits*

As a means of supplying acute protein deficits following surgical shock and hemorrhage, amino acids have the same disadvantage as simple solutions of glucose and saline in that they possess no colloidal properties. Before they can do so they must be synthesized into plasma protein, which takes time. On the other hand, experimental evidence indicates that solutions containing amino acids exert a more beneficial effect after hemorrhage than solutions of glucose or saline alone (26). Clinical observations have not been made.

### 3. *Amino Acids as Parenteral Protein Food*

The therapeutic use of amino acids as a part of parenteral alimentation has become quite extensive. Amino acids as a source of protein food are quite similar to glucose as a source of carbohydrate food. Appropriate mixtures of amino acids as such or as hydrolyzed protein, therefore, represent a physiological method for introducing protein nourishment outside of the gastrointestinal tract. The general indications for the use of parenteral nourishment may be summarized in three groups: (1) To correct cumulative deficits in chronic depletion. (2) To prevent protein starvation after operation by meeting the daily needs for protein. (3) To supplement the normal oral intake.

(1) The correction of chronic deficits in malnourished patients who have for a long period of time been unable, because of obstruction, to eat normally, presents a large, quantitative problem, inasmuch as these deficits are cumulative. For example, a patient who has lost 50 pounds of body weight has probably lost at least a third of it as protein tissue, or in terms of dry protein, 3000 g. The complete replacement of all of this amount of protein through the intravenous injection of amino acids and plasma, while theoretically possible, is so difficult that thus far, at least, no one apparently has tried to do so. In actual practice it may not be necessary to replace all of these losses completely. Certainly, the clinical benefit which follows even partial correction is considerable. A reasonable amount of parenteral protein nourishment can be given to these patients while other preparations are made for operation. Commonly about twice what might be called the minimum daily need, *i.e.*, about 100 to 150 g. per day is a convenient amount. This represents in the case of Amigen 2 or 3 l. of a solution containing 5% (plus 5% glucose). Such a regime plus an occasional plasma or whole blood transfusion will do much to correct these cumulative deficits and certainly prepare

the patients for operation much more effectively than has been possible heretofore. Regarding larger amounts, experimental evidence has shown that in the dog the intravenous injection of 10 g. of amino acids per kilogram per day can be utilized. This, in terms of a normal sized adult, would be 700 g. or 14 l. of 5% Amigen a day. Until clinical study has shown that such a large intake is without deleterious effects, the more moderate dose just mentioned should be used.

For the correction of such chronic deficits one obviously means the rebuilding of tissue protein, *i.e.*, cell protoplasm. Potassium and magnesium salts are essential constituents of cells and their addition to the parenteral diet may prove of value in promoting cellular protein synthesis. Other less obvious factors may also prove important, but await further study.

(2) The second general indication for the use of amino acids is for the prevention of protein starvation during the postoperative period, when the patient cannot eat. This can be achieved by meeting the daily requirements of protein nourishment. Under the simplest conditions one liter of fluid containing 5% Amigen (plus 5% glucose) would be considered a minimum requirement. However, after severe operations the loss of nitrogen is great and there is certainly a greater need for protein. At least 2 l. are therefore indicated, particularly when a deficiency was present before the operative procedure. It is not always possible to maintain nitrogen balance after operation with this amount of amino acids, or indeed, even with larger doses. On the other hand, it may be questioned whether nitrogen balance is necessary or even advisable in such cases. Complete is obviously worse than partial protein starvation and this can be avoided in most cases by the administration of amino acids from the very beginning of treatment. The clinical benefits have been striking even when moderate amounts have been so injected. This is particularly true when other elements, including vitamins, plasma and whole blood, have been added to the parenteral regime.

(3) Supplementary injections of amino acids may occasionally be employed to augment a deficient oral intake of protein. *A priori* one might state that tube feeding in such cases would be preferable to intravenous feeding. However, many patients object strenuously to tube feeding, or vomit when this method of alimentation is used. In such a situation it may be advisable to supplement the oral intake by intravenous feeding. Not infrequently supplementation of this kind for only a few days will so improve the nutrition of the patient that a gradually increasing diet will be taken by mouth, particularly when parenteral vitamins also are injected.

When intravenous feeding is used to supplement oral intake an im-

portant detail is the time selected for the injection. No patient, particularly those who are unable to take much food by mouth, will wish to eat while an intravenous injection is going on, whether it be glucose, saline, plasma, blood or amino acids. For this reason, supplementary injections should always be made between meals or better still, after the evening meal when the patient has taken as much as he will by mouth. Ordinarily, the addition of 50 g. each of protein and carbohydrate food is sufficient and this is readily done by giving one liter of Amigen 5%, glucose 5%, in the evening between the hours of 5 and 8 or 7 and 9.

## VI. SUMMARY

Present knowledge has been briefly reviewed of the possible dangers as well as the metabolic behavior of intravenously injected protein as human plasma and protein hydrolyzates, including pure amino acid mixtures. Both are now available as safe preparations which can be given with little or no danger. The evidence shows that while nitrogen balance can be achieved during plasma transfusions, the metabolic behavior of protein given in this way is still far from understood. The clinical value of plasma to correct acute deficits is clear and established. As a source of parenteral protein food plasma has clinical value, but its mechanism rests on much less secure knowledge.

The metabolic behavior of appropriate amino acid mixtures as protein hydrolyzates injected intravenously is fairly well known and probably differs little from the same substances absorbed from the gastrointestinal tract. Much more knowledge is needed of its relation to other nutriment, and of the effect of hydrolyzates containing larger aggregates of amino acids. Its clinical value as a source of parenteral protein food seems well established.

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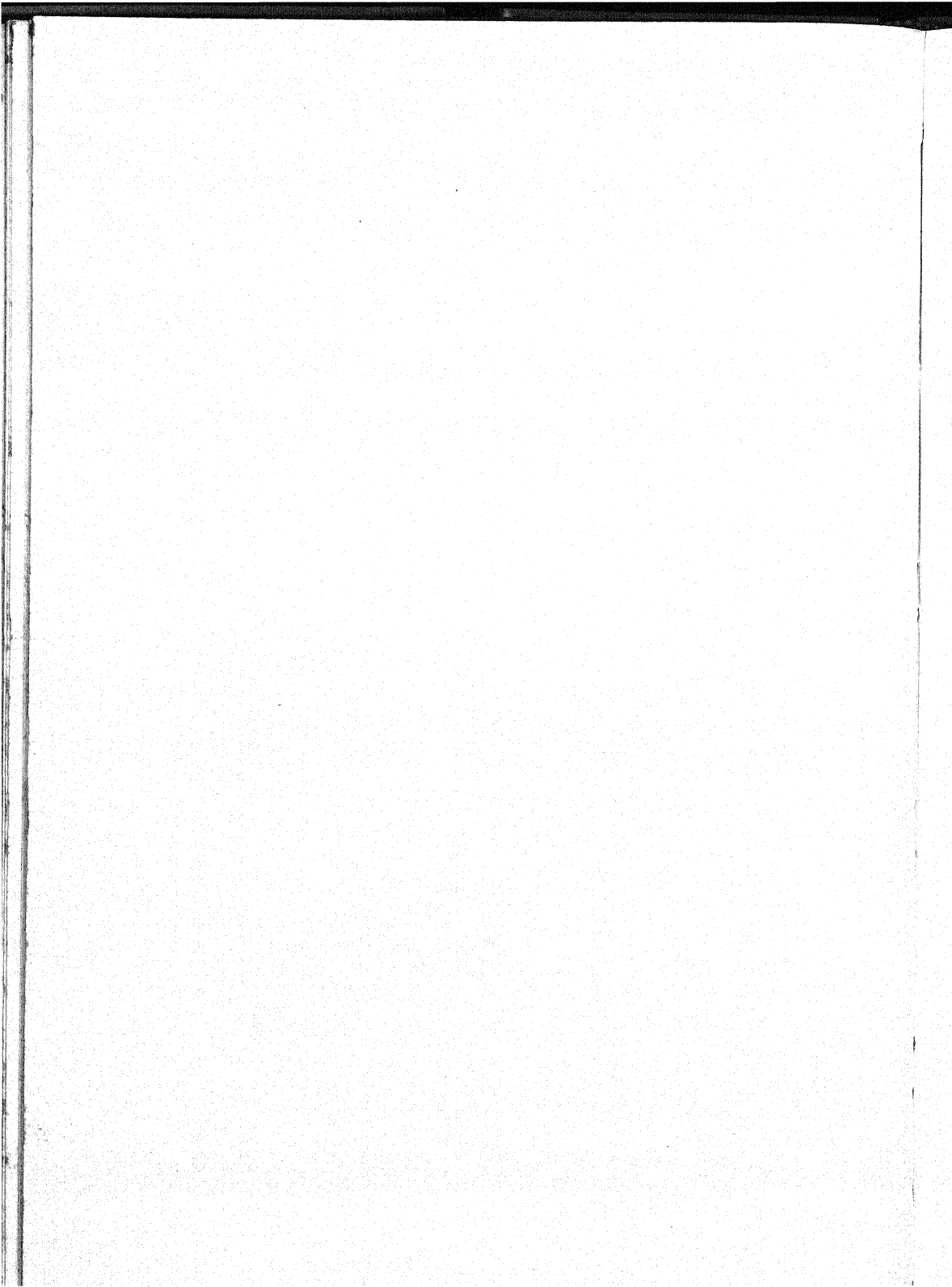
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# The Preparation and Criteria of Purity of the Amino Acids

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## I. INTRODUCTION

This brief outline of methods for the preparation, purification and determination of purity of the commonly occurring amino acids is presented because of its possible usefulness to investigators who may require pure amino acids for experimental purposes. Previous reviews (110a, 138, 156, 163, 203, 780) have been concerned, primarily, with the theoretical rather than the practical aspects of this topic. Consideration has been given in the present discussion only to the DL-, L-, and D-forms of the 21 amino acids which have been shown to be constituents of proteins.\*

Amino acids may be synthesized by the following type reactions:

Amination of  $\alpha$ -halogen acids, unsaturated dicarboxylic acid esters and anhydrides.

Hydrolysis of aminonitriles prepared by reaction of ammonia and hydrocyanic acid with aldehydes (Strecker reaction).

Hydrolysis of the acyl, aryl or quaternary ammonium derivative of malonic ester, acetoacetic ester or cyanoacetic ester and of the phthalimido, benzamido, acetamido, or  $\alpha$ -oximino derivative of these esters.

Hydrolysis of the products prepared by the reaction of aldehydes with hippuric acid, hydantoins or diketopiperazines.

Catalytic reduction of oximes and phenylhydrazones of  $\alpha$ -keto acids.

Catalytic reduction and amination of  $\alpha$ -keto acids.

Beckmann rearrangement and hydrolysis of cycloketoximes followed by bromination and amination of the resulting  $\omega$ -aminomonocarboxylic acids.

Amination and hydrolysis of  $\alpha$ -bromo- $\beta$ -methoxy acids. The latter are prepared from the methanol and basic mercuric acetate or the bromine addition products of unsaturated monocarboxylic acids.

Oxidation and hydrolysis of benzoylamino alcohols.

Reaction of hydrazoic acid and  $\alpha$ -amino acids (Schmidt synthesis of diamino acids).

Addition of sodium enol malonates to  $\alpha,\beta$ -unsaturated acid esters (Michael condensation).

Degradation of substituted malonic acid hydrazides (Curtius reaction).

Isolation procedures employed for the preparation of L-amino acids vary with the amino acid and the starting material. The proportions of amino acids in gelatin, blood, wheat protein, casein, silk, corn protein, plant sprouts, human hair, rabbit fur, fish protein and other materials commonly used for this purpose differ so markedly and the solubility relations of the amino acids in the hydrolyzates of these products vary so widely that the development of satisfactory isolation procedures has been a special problem for each amino acid and for each protein.

\*The capital L- and D-terminology employed in this paper refers to the relative configuration of the amino acids.

Some amino acids are readily separated from hydrolyzates of certain proteins by crystallization at their isoelectric points. The ones most commonly isolated in this manner are glutamic acid and leucine from cereal grains, leucine and tyrosine from blood proteins and cystine from hair and other keratins. Arginine, histidine and lysine are often removed by precipitation as their silver salts of phosphotungstates, by electrodiolysis, by ion exchange (110) or by selective adsorption. Other group separations sometimes employed advantageously include fractional distillation of the ethyl esters of the low-molecular weight amino acids, fractional crystallization of the barium or barium carbamate salts of aspartic and glutamic acids and of the copper salts of various amino acids, and the extraction of proline and the monoamino monocarboxylic acids from aqueous solution with butanol. A list is given below of special amino acid salts which have been employed under selected conditions of amino acid and salt concentration, amino acid-salt ratio, solvent, pH and temperature.

Alanine pyridoxate (dioxalatodipyridinochromiate) and azobenzene-*p*-sulfonate.

Arginine flavianate (1-naphthol-2,4-dinitro-7-sulfonate) and picrate. Aspartic acid copper salt trihydrate.

Glycine ethyl ester hydrochloride, trioxalatochromiate and 5-nitro-naphthalene-1-sulfonate.

Histidine dihydrochloride, mercuric chloride and mercuric sulfate complexes, and 3,4-dichlorobenzene sulfonate.

Hydroxyproline reineckate.

Leucine 2-bromotoluene-5-sulfonate and  $\beta$ -naphthalene sulfonate.

Lysine monohydrochloride, dihydrochloride and picrate.

Methionine mercuric chloride and mercuric sulfate complexes.

Phenylalanine 2,5-dibromobenzene sulfonate.

Proline rhodanilate.

Serine *p*-hydroxyazobenzene-*p'*-sulfonate.

Tryptophan mercuric sulfate complex.

The DL-forms of nearly all of the amino acids have been prepared by racemization. The optically active amino acid is heated with a strong base, strong acid or an alkaline solution of acetic anhydride or ketene. Practical difficulties are that only a few of the optically active amino acids are readily obtainable, high temperatures and pressures may be required to effect rapid racemization and some amino acids are partly or wholly decomposed by treatment with hot acid or alkali.

Most of the amino acids have been resolved by fractional crystallization of the salts formed with optically active bases such as quinine, brucine, strychnine and cinchonine and the formyl or benzoyl derivatives of the L- and D-isomers of the amino acids present in DL-mixtures (291).

Alanine, valine, and phenylalanine have been resolved through their menthoxyacetyl derivatives (406) and lysine has been resolved with the aid of its camphoric acid salt (83). It has been shown that the natural optical isomers of amino acids may be obtained as their anilides by the action of aniline and papain on N-derivatives of the DL-forms (87, 334). Advantage has also been taken of the specificity of yeasts (234, 236), molds, bacteria, and enzymes for one of the two optical forms of amino acids to obtain the isomer not attacked, or utilized more slowly than the other antipode. A practical method of this kind has been described by Duschinsky and Jeannerat (229) and Behrens (79) who prepared the L-isomers of alanine, isoleucine, methionine and valine by oxidizing the D-isomer of the DL-mixtures with D-amino acid oxidase obtained from pig kidney.

## II. SYNTHESIS AND ISOLATION

### 1. *Alanine*

*Synthesis.* The method described by Tobie and Ayres (783, 784) is satisfactory. Commercial DL- $\alpha$ -bromopropionic acid<sup>1</sup> is aminated with a large excess of ammonia to minimize the formation of  $\alpha$ -iminodipropionic acid and  $\alpha$ -N-tripropionic acid. The yield of product twice recrystallized from aqueous-methanol is about 120 g. (67%) from two moles of the bromoacid. The latter is aminated conveniently in a 12-l. flask.

DL-Alanine may be prepared conveniently and inexpensively by the Strecker reaction. By the procedure of Kendall and McKenzie (452) a cold ether solution of freshly distilled acetaldehyde, ammonium chloride and sodium cyanide are shaken for four hours at room temperature in a tightly-stoppered ginger-ale bottle. The solution is acidified and hydrolyzed, the crude DL-alanine hydrochloride is dissolved in 95% ethanol and the chloride ions are removed from the final product by treatment with yellow lead oxide and silver oxide. Approximately 300 g. of crude DL-alanine may be prepared by a modified procedure in which an ice-cold solution of sodium cyanide is added dropwise into an ice-cold suspension of acetaldehyde, ammonium chloride, water and diethyl ether contained in a 5-l. round-bottom three-necked flask equipped with a reflux condenser, a mercury-sealed motor stirrer and a dropping funnel and immersed in an icebath. The three-phase mixture is stirred for four hours, acidified and hydrolyzed. The crude product, obtained by evaporation of the mixture, is dissolved in 3 l. of methanol, the solution is brought to pH 7 by the addition of ammonium hydroxide, the suspension is filtered and the resulting DL-alanine is washed thoroughly with methanol.

By the Strecker method of Cocker and Lapworth (162) about 200 g.

<sup>1</sup> see p. 359.

of crude DL-alanine are obtained when four moles of acetaldehyde are added with stirring to eight moles of ammonium hydroxide and six moles of anhydrous hydrocyanic acid<sup>2</sup> are added with stirring to the acetaldehyde-ammonia solution. This mixture is stirred for 45 minutes, 24 moles of HCl are added, the acid solution is boiled for 3 hours and an equal volume of methanol is added to the residual solution. The suspension is cooled and filtered, and the precipitate is washed thoroughly with methanol.

Another practicable synthesis of alanine has been described recently by Billman and Parker (97, 99). Alanine is prepared in 45% of the theoretical yield by permanganate oxidation of commercially available 2-benzoylamino-1-propanol<sup>3</sup> and acid hydrolysis of the resulting benzoyl-alanine. Other syntheses which are of value for special purposes, such as the introduction of isotopic atoms into amino acid molecules, include the catalytic reduction of the phenylhydrazone of pyruvic acid (264, 272, 298) and of  $\alpha$ -oximinopropionic acid (366, 718).

DL-Alanine has been prepared by racemizing L-alanine with Ba(OH)<sub>2</sub> (296) and with a mixture of glacial acetic acid and acetyl chloride (852) or acetic anhydride (178). N-Benzoyl-DL-alanylchloride has been prepared by racemizing N-benzoyl-L-alanylchloride with PCl<sub>5</sub> (444).

*Isolation.* The best natural source of alanine is silk which contains nearly 25% of this amino acid. Other proteins contain less than 10% of alanine. Alanine may be readily isolated by the classical ester procedure of Emil Fischer (280) which has been applied to silk, silk fibroin, sericin, casein, gelatin, and other proteins. Glutamic acid hydrochloride is crystallized from the protein hydrolyzate, the remaining amino acids are esterified and glycine ethyl ester hydrochloride is precipitated. The free amino acid esters are fractionally distilled *in vacuo* and the fraction containing alanine ethyl ester (b. p. about 50°C./10 mm.) is hydrolyzed. The yield (308) of nearly analytically pure L-alanine (225) is about 50 g. (20%) from 250 g. of silk fibroin.

A satisfactory isolation procedure has been described recently by Stein *et al.* (755). After refluxing silk fibroin for 8 hours with concentrated hydrochloric acid, the bulk of the acid is removed by vacuum distillation and treatment with lead acetate. Tyrosine is removed, the concentrated filtrate is treated with a solution of 5-nitronaphthalene-1-sulfonic acid dihydrate<sup>4</sup> and the slightly soluble glycine naphthalene sulfonate is removed. Methyl cellosolve and a solution of azobenzene-*p*-sulfonic acid trihydrate<sup>5</sup> are added, the suspension is filtered and the precipitate of L-alanine azobenzene sulfonate is treated with barium acetate. The suspension of barium azobenzene sulfonate is filtered and the L-alanine obtained from the filtrate is recrystallized from aqueous ethanol

<sup>2-5</sup> see p. 359.

The yield of nearly pure L-alanine is 24 g. (22%) from 108 g. of technically degummed silk.

By the method of Bergmann and Niemann (92), glycine is removed from an acid hydrolyzate of silk fibroin as its trioxalatochromiate, and alanine is precipitated with a solution of sodium dioxypyridate.<sup>6</sup> The yield from 31.6 g. of silk fibroin is 43.1 g. of alanine dioxypyridate equivalent to 7.8 g. (24.6%) of L-alanine. It seems probable that the largest part of the alanine could be recovered from its dioxypyridate.

The isolation of alanine from silk fibroin as its hydantoin (119) and from gelatin (505) and gluten (188) by a complicated copper salt procedure has been reported.

## 2. Arginine

*Synthesis.* Schulze and Winterstein (706, 708) prepared 1 g. of arginine nitrate by allowing a solution of cyanamide and ornithine to evaporate over sulfuric acid for three weeks; while Sørensen *et al.* (747, 750) synthesized arginine nitrate by the reaction of cyanamide and  $\alpha$ -benzoylamino- $\delta$ -amino-*n*-valeric acid. Ornithine is readily synthesized from cyclopentanone by the methods described by Fox *et al.* (328) and Albertson and Archer (47) and ornithuric acid ( $\alpha,\delta$ -dibenzoylamino-*n*-valeric acid) is easily prepared by the benzylation of ornithine. The other steps in the described syntheses of arginine are less satisfactory since they involve either the selective reaction of cyanamide with the  $\delta$ -amino group of ornithine or the selective hydrolysis of the  $\delta$ -benzoyl group of ornithuric acid.

DL-Arginine nitrate (490) and carbonate (666) have been prepared by racemizing L-arginine carbonate with  $H_2SO_4$ . Monoacetyl-DL-arginine has been prepared by racemizing the corresponding L-form with acetic anhydride (96, 840), acetyl chloride (852), and ketene (582).

*Isolation.* About 75 g. (6.2% yield of free arginine) of nearly pure L-arginine monohydrochloride are readily prepared from 1 kg. of purified gelatin by Cox's (174) modification of Kossel and Gross's (479) method. Smaller yields are obtained from ground-hide glue (low grade gelatin). Gelatin is hydrolyzed with HCl, the excess acid is distilled *in vacuo* and an aqueous solution of flavianic acid<sup>7</sup> is added to a solution of the sirup. The resulting L-arginine monoflavianate is decomposed with HCl, the suspension of flavianic acid is filtered, and aniline<sup>8</sup> is added to the concentrated filtrate. The crystalline L-arginine monohydrochloride is purified by treatment with norite and recrystallization from aqueous-ethanol solution. Unless arginine is precipitated initially at about pH 4, a mixture of the mono- and diflavianates may crystallize. Other modifications of Kossel's procedure, including preliminary electrodialysis (177, 323,

<sup>6-8</sup> see p. 359.

324), have been described for the isolation of L-arginine from gelatin (263, 335, 440, 643), hemoglobin (90, 177, 825), human hair (826), fibrin (89), protamines (477, 480), salmon muscle (63) and other proteins.

Other types of salts which have been employed for the isolation of L-arginine include the silver salt (477, 480, 822, 823), the picrolonate (323, 324), the silver nitrate salt (335, 477), the nitrate (358), and the benzylidene derivative (95, 124). As described by Fox (327), L-arginine may be readily prepared by treating a solution of L-arginine monohydrochloride with silver oxide, filtering the suspension of AgCl and evaporating the filtrate *in vacuo* in a stream of CO<sub>2</sub>-free air.

### 3. Aspartic Acid

*Synthesis.* DL-Aspartic acid is prepared in about 60% of the theoretical yield by the method of Dunn and Fox (208). A mixture of dry ammonia and diethyl fumarate<sup>9</sup> dissolved in absolute ethanol is placed in an autoclave or tightly stoppered thick-walled bottle and heated with boiling water or steam at atmospheric pressure for 24 hours. The resulting crude diketopiperazine diacetamide is refluxed for 6 hours with 6 *N* NaOH, a solution of copper acetate monohydrate<sup>10</sup> is added and the resulting suspension of crystalline cupric aspartate trihydrate is filtered. The crude copper salt is decomposed with H<sub>2</sub>S, and aspartic acid is crystallized from the filtrate. It has been found that the synthesis may be carried out more conveniently with commercially available maleic anhydride as starting material. Maleic anhydride is heated with aqueous ammonia in an autoclave, the residual intermediate solids are hydrolyzed by heating them with concentrated HCl, crystalline NH<sub>4</sub>Cl is removed from the concentrated filtrate, and DL-aspartic acid is crystallized at pH 3.

The syntheses of aspartic acid by (a) the reaction of ethyl chloroacetate with ethyl sodium phthalimidomalonate (222) and with ethyl benzamidomalonate (660) and (b) the catalytic reduction of ethyl- $\alpha$ -oximinosuccinate (366, 849) are satisfactory but less convenient than the described direct amination procedures. Other starting substances which have been employed for the synthesis of aspartic acid include ammonium malate (193, 874), oxalacetic acid (470) and oxime ester (637), hydroxylaminofumaric acid (775), ethyl acetylsuccinate (687), ethyl bromosuccinate (473), bromosuccinic acid (303, 843), ethyl dicarbitetracarbonate (632) and ethyl aminomalonate (445). DL-Aspartic acid has been prepared by racemizing the L-form. The methods reported include heating L-aspartic acid with 3.5 *N* HCl for 3 hours at 180°C. (238, 569, 570, 807), with excess Ba(OH)<sub>2</sub> solution for 10 hours at 145°C. (807) and with a mixture of glacial acetic acid and acetyl chloride (853) for four hours at 100°C. In the latter case it was necessary to heat the

<sup>9, 10</sup> see p. 359.



product with 10% HCl for 3 hours at 100°C. to complete the racemization.

*Isolation.* L-Aspartic acid is readily prepared from natural asparagine in nearly the theoretical yield by Vickery and Pucher's (827) modification of Schiff's (680) method. A mixture containing 3.9 ml. of 3.5 *N* HCl per g. of natural asparagine monohydrate is refluxed for 3 hours, 3.5 *N* NH<sub>4</sub>OH is added to bring the solution to pH 3, and 2 volumes of 95% ethanol are added. The resulting crystalline product is recrystallized from boiling water.

Vickery and coworkers have described a practicable procedure for the isolation of natural asparagine from the etiolated seedlings of *Lupinus albus*<sup>11</sup> (828) and *Lupinus angustifolius*<sup>11</sup> (827). The seedlings are grown in coarse wire-mesh trays or baskets placed in a light-tight cabinet. Nearly 100% humidity and 20–25° temperature are maintained. All equipment is scrupulously cleaned and sterilized before use. Germination occurs within 2 or 3 days. The seedlings are allowed to grow as long as the plants appear healthy and the content of amide nitrogen increases (20 to 30 days for *L. albus*, and 12 to 16 days for *L. angustifolius*). Without discarding the testa<sup>12</sup> tissue, the moist sprouts and roots are macerated in a Waring blender or ground in a meat grinder with the aid of dry filter paper (or the seedlings are dried at 80°C. in a current of air, and the dried material is ground to a fine powder in a Wiley mill). The mass of moist material is filtered with the aid of a screen, and the pulp is pressed in a hydraulic press or by hand. The combined filtrates and press juice are heated to 80–90°C. with steam to coagulate the protein, acetic acid is added to pH 6, the suspension is allowed to stand overnight, and the supernatant liquid is filtered through pulp covered with celite. The residual pulp is washed, the combined liquids are concentrated *in vacuo*, and the sirup is seeded with crystals of asparagine. The resulting crude asparagine hydrate is washed and recrystallized from about 5 volumes of distilled water. The yield of nearly pure product from *L. albus* is about 15%, and from *L. angustifolius* about 9% of the dry weight of the seeds.

It has been found<sup>13</sup> that yields of asparagine essentially the same as that reported by Vickery *et al.* may be obtained either by growing the seeds in moist-sand trays placed in a dark cabinet or by growing them outside in the ground. In the latter case, the bed was made light tight by means of a cover resting on walls about 10 inches high. The seedlings were not sprayed with water at any time, and no difficulty with fungus growth was encountered. After 12 to 14 days growth, the weight of the moist seedlings was about ten times that of the seeds. The yield of purified asparagine hydrate was about 10% of the dry weight of the seeds.

The method described by Vickery *et al.* for the isolation of asparagine

<sup>11-13</sup> see p. 359.

was essentially the same as that employed previously except that early workers usually removed proteins and other impurities from the concentrated extracts of seedling sprouts by precipitation with lead acetate. Although asparagine was first isolated from asparagus juice by Vacquelin and Robiquet (806) in 1806, asparagus sprouts contain only about 0.2% asparagine according to Winterstein and Huber (872). The methods employed by Piria, Schulze and other early workers in the isolation of asparagine from seedlings (particularly of the vetch and lupine species) and the data<sup>14</sup> reported by these investigators, have been reviewed by Winterstein (871), Vickery and Schmidt (829) and Chibnall (150). More recently, Vickery and coworkers (816, 817, 827, 828) have investigated the amide and asparagine content of alfalfa and the seedlings of the soybean, *Vicia atropurpurea* (purple vetch), *Vicia villosa*, *Vicia faba* (broad or horse bean), and *Cucurbita pepo* (summer squash). Asparagine has been isolated from the poppy, sunflower, beet, potato and numerous other plant species listed by Steele (753) and Onslow (599). According to Harvey (381), the production of asparagine is greatly increased in plants grown in air containing ethylene.

L-Aspartic acid has been isolated in relatively small quantities from numerous proteins by methods which have been employed primarily for analytical purposes. The isolation of aspartic acid as barium-DL-aspartate, first described by Fischer (280) in 1901, has been applied to different proteins by Fischer, Abderhalden, Osborne, Jones and other workers as recently as 1928 (488) even though, in 1910, Osborne and Jones (616) were able to recover only 42% of aspartic acid from a mixture of pure amino acids. By Foreman's (318) modification of the Ritthausen (669, 670) procedure, calcium aspartate and glutamate are precipitated with calcium hydroxide and ethanol, calcium is removed from the precipitate as the oxalate and aspartic acid is isolated as the copper salt. Jones *et al.* (419, 420, 431, 433-435), Bergmann and Niemann (89, 90), and Chibnall *et al.* (151) have isolated aspartic acid from various proteins by this method. Other investigators have employed for this purpose various combinations of barium, lead and copper aspartates.

#### 4. Cystine

*Synthesis.* DL-Cystine is prepared in about 23% over-all yield by the method of Wood and du Vigneaud (877). S-Benzylthiolmethylphthalimidomalonic ester, prepared from benzylthiolmethyl chloride<sup>15</sup> and sodium phthalimidomalonic ester, is hydrolyzed with NaOH to S-benzyl-DL-cysteine. The latter substance is reduced with sodium in liquid ammonia, and the resulting DL-cysteine is oxidized with air (catalyzed by

<sup>14,15</sup> see p. 359.

$\text{FeCl}_3$ ) to DL-cystine. Nearly the same yield of DL-cystine was obtained by Tarver (776) by analogous reactions of methylthiolmethylphthalimidomalonic ester. This substance was hydrolyzed first with NaOH and then with HCl to S-methylcysteine which was demethylated by boiling it for 6 hours with concentrated HI solution. The following less practicable syntheses of cystine have been described. Erlenmeyer (255) and Erlenmeyer and Stoop (260) prepared benzoylcysteine ethyl ester from benzoylserine ethyl ester and  $\text{P}_2\text{S}_5$ , hydrolyzed the product with HCl and oxidized the resulting cysteine in the presence of ammonia and  $\text{FeCl}_3$ . Fischer and Raske (305) prepared  $\alpha$ -amino- $\beta$ -chloropropionic acid hydrochloride from serine ethyl ester and  $\text{Ba}(\text{SH})_2$ , removed the barium ions from the solution as  $\text{BaSO}_4$ , and oxidized the cysteine with air.

DL-Cystine may be prepared by racemizing L-cystine with HCl. A mixture containing about 10 ml. of 6 to 12 N HCl per g. of L-cystine is refluxed for four to six days and the resulting DL-cystine is purified by dissolving it in HCl, reprecipitating it at the isoelectric point, and recrystallizing it from 6.5 N HCl. This procedure described by du Vigneaud *et al.* (405, 529, 834) is essentially that originated by Mörner (574, 575) in 1899. Other modifications of Mörner's method have been reported by Neuberg and Mayer (585), Hoffman and Gortner (403), and Gortner and Sinclair (350). Toennies and Elliot (787) racemized L-cystine by refluxing for 40 hours a mixture containing 3.5 ml. of 54%  $\text{H}_2\text{SO}_4$  per gram of amino acid. Studies have been reported showing the rate of racemization of L-cystine in boiling 6.5 N HCl (403) and in 2.5 N solutions of HCl,  $\text{H}_2\text{SO}_4$  and  $\text{H}_3\text{PO}_4$  at 38° and 60°C. (54). The purification and some physical properties of L-, D-, DL- and mesocystine have been investigated particularly by Andrews and de Beer (56), Toennies and Lavine (789) and Loring and du Vigneaud (528, 529). The attempts to prepare DL-cystine by heating the natural isomer with glacial acetic acid and acetyl chloride (852), acetic anhydride and NaOH (840, 841) or ketene (135) were unsuccessful. Wood and du Vigneaud (876) prepared S-benzyl-DL-cysteine in good yield by racemizing S-benzyl-L-cysteine with acetic anhydride and NaOH. Presumably this product could be de-benzylated and the DL-cysteine oxidized to DL-cystine. The preparation of DL-cystine by heating the natural antipode with alkali is not attempted since, as noted by Neuberg and Mayer (585) in 1905, this amino acid is decomposed under these conditions to  $\text{H}_2\text{S}$ , S,  $\text{NH}_3$ , pyruvic acid and other products listed by Thor and Gortner (782), Lindstrom and Sandstrom (518), Thomas and Hendricks (781) and other workers referred to by these authors. Routh (674) found that  $\text{H}_2\text{S}$ , cysteine and sulfur are formed in appreciable quantities even when cystine is boiled in air-free water for periods up to 48 hours. Andrews (55) has shown that

the rotation of an alkaline solution of cystine decreased to zero after standing for 128 weeks under nitrogen.

*Isolation.* L-Cystine is readily isolated in about 5% yield essentially by Mörner's (574) method.<sup>16</sup> Hair, obtained from a men's barber shop, is separated from foreign materials, extracted with cold cleaning solvent by suspending and stirring it in this liquid, and dried in air. A mixture containing about 3 ml. of 8 N HCl per g. of purified hair is refluxed for 8 hours, the suspension of humin is filtered, and the filtrate is decolorized with sufficient norite or Nuchar to remove most of the color. The filtrate is brought to pH 4 by the addition of concentrated NaOH, the mixture is allowed to stand overnight in the refrigerator and the suspension of L-cystine is filtered. The crude product is purified by dissolving it in 3 N HCl, reprecipitating it at the isoelectric point and washing the precipitate thoroughly with boiling distilled water to remove last traces of tyrosine and other impurities.

L-Cystine has been isolated essentially by this procedure by Folin (316), Gortner and Hoffman (348), Vickery and Leavenworth (826), Toennies and Bennett (786), Lucas and Beveridge (532), and other workers referred to by these authors. The experimental variations included differences in the time and temperature of hydrolysis, type of acid catalyst, type of alkali employed to neutralize the acid, and methods used to purify the crude products. L-Cystine is usually isolated at its isoelectric point (pH 4-5) although procedures have been suggested for precipitating it as its phosphotungstate (638, 787, 870), HgSO<sub>4</sub> complex (380, 407, 602, 607, 625), bis 3,4-dichlorobenzenesulfonate (830), copper salt (354, 786), and cuprous cysteine derivative (532, 658). Yields of cystine considerably higher than 5% have been reported by some workers but few, if any, of the preparations were unquestionably pure L-cystine. Most workers have neglected to report either the degree of purity or to employ reliable criteria for the determination of purity. Although the specific rotation of pure L-cystine (see Table VI) is a dependable criterion of purity, account must be taken of the temperature coefficient,  $-2.04^{\circ}$  (209), in measurements made at temperatures other than that used as standard. Although the hair from different species of animals and from humans of different sexes, ages and races varies widely in total cystine content according to literature reports (160), values as high as 18% have been found by colorimetric analysis. It may be reasonable to expect, therefore, that more than one third of the total cystine may be obtainable as pure L-cystine by improved procedures.

### 5. 3,5-Diiodotyrosine

*Synthesis.* 3,5-Diiodotyrosine is prepared in about 80% yield by the method of Block and Powell (103). A suspension of tyrosine in acetic

<sup>16</sup> see p. 359.

acid is dissolved in a solution of ICl in acetic acid, the solution is maintained at 60°C. while about 1.5 volumes of water are added,  $\text{NaHSO}_3$  is added sufficient to reduce excess ICl, ammonia is added to precipitate the 3,5-diiodotyrosine, and the crude product is recrystallized from dilute HCl. Other modifications (14, 74, 370, 393, 550, 618, 679, 886) of the method originated by Wheeler and Jamieson (860) have been reported. Li (515) has proposed a probable mechanism of this synthesis. According to Abderhalden and Guggenheim (14) the specific rotation of 3,5-diiodotyrosine in 4% HCl is  $[\alpha]_D^{20} = +2.9^\circ$ . Harington and Randall (375) reported the value,  $[\alpha]_D = +2.8^\circ$ .

*Isolation.* 3,5-Diiodotyrosine was first isolated by Drechsel (202) in 1896 from the alkaline hydrolyzate of the horny skeleton of the coral *Gorgonia cavolinii*. This amino acid has been isolated from gorgonia skeletons (392, 393, 622, 769), bath sponges (41, 861), iodinated proteins (619-621), thyroid (374) and thyreoglobulin (320, 375).

### 6. Glutamic Acid

*Synthesis.* The most convenient synthesis is that described by Marvel and Stoddard (558). DL-Glutamic acid is prepared in about 75% of the theoretical yield by the reaction of methyl acrylate, ethylphthalimidomalonate and sodium in absolute ethanol. The reaction mixture is concentrated, the residue is refluxed with 6 N HCl, the suspension of phthalic acid is filtered, the filtrate is decolorized and brought to pH 3, and the resulting crude glutamic acid is recrystallized from aqueous ethanol as the monohydrate. The closely related synthesis from methyl acrylate, diethyl acetamidomalonate<sup>17</sup> and sodium has been reported by Snyder *et al.* (740). DL-Glutamic acid may also be synthesized satisfactorily by the reaction of ethyl  $\beta$ -bromopropionate with ethyl benzamidomalonate (223, 660) and by the catalytic reduction of ethyl  $\alpha$ -oximinoglutarate (366, 542). The latter substance may be prepared from sodium, ethyl acetoacetate and ethyl  $\beta$ -chloropropionate. Less practicable methods for the synthesis of DL-glutamic acid from levulinic acid (875), diethyl diazoglutarate (153),  $\alpha$ -ketoglutaric acid (470) and acrolein (446) have been reported.

The monohydrate, the hydrochloride and the anhydrous forms of DL-glutamic acid are readily prepared by modifications (61, 224) of the method of Abderhalden and Kautzsch (19). L-Glutamic acid is heated at about 200°C. for 4 hours, the molten mass is dissolved as completely as possible in 6 N HCl, the suspension is filtered, and the filtrate is refluxed 4 hours. Concentrated HCl is added, the resulting suspension of crystals is filtered and the crude DL-glutamic acid hydrochloride is recrystallized from 6 N HCl. The monohydrated and anhydrous forms

<sup>17</sup> see p. 359.

are prepared as described by the authors quoted. Factors influencing the glutamic acid-pyrrolidonecarboxylic acid system, first investigated by Haitinger (364), have been studied by Wilson and Cannan (865) and other works referred to by the latter authors.

L-Glutamic acid is partially racemized by heating it with HCl (60, 421). It is completely racemized by long-continued heating with  $\text{Ba}(\text{OH})_2$  at  $120^\circ\text{C}$ . to  $170^\circ\text{C}$ . (62, 300, 702) and by heating its N-acetyl derivative for a short time with glacial acetic acid and acetic anhydride (96) or acetyl chloride (852) and with NaOH and acetic anhydride (840) or ketene (135).

*Isolation.* L-Glutamic acid is isolated commercially in large quantities from wheat gluten and the waste water (Steffen's waste)<sup>18</sup> from the manufacture of beet sugar. Monosodium L-glutamate is an important condiment since, even in low concentrations, it imparts a meat-like flavor to food materials. L-Glutamic acid is usually isolated as the hydrochloride from the HCl hydrolyzate of wheat gluten, averaging about 30% glutamic acid, and of concentrated Steffen's waste (sp. gr. 1.4) containing from 1 to 6% of glutamic acid. More than 100 patents have been issued on the isolation of glutamic acid, the first (U. S. 1,015,891) to Okeda and Suzuki of Tokyo in 1912 and the last (U. S. 2,380,890) to V. S. Waters of Minneapolis in 1945.

In the laboratory procedure<sup>19</sup> described by Anslow and King (57, 458) gluten flour is refluxed 6 hours with concentrated HCl, the solution is decolorized, and the suspension is filtered. The filtrate is concentrated *in vacuo* and allowed to stand at low temperature. The combined first and second crops of L-glutamic acid hydrochloride are recrystallized from 6 N HCl. The yield of this product is 26–27%. Yield as high as 41–43% have been obtained from gliadin (612) and hordein (467) and 31% from zein (184). Less than 20% of L-glutamic acid has been isolated from most protein materials including the pea (608, 613–615), soybean (606), almond (609), peanut (419), fibrin (89), castor bean (443), pumpkin seed (8), kidney bean (603), squash seed (610), sunflower seed (26), wool (488), cotton seed (28), lactalbumin (434), hen's eggs (20), gelatin (183), Brazil nut (610), cocoanut (420), keratins of cattle and horses (11), and the cat, rabbit and chicken (whole animals except feathers or fur and intestines) (13). The preparation of L-glutamic acid from monosodium L-glutamate obtained commercially has been described by Schmidt and Foster (685).

### 7. Glycine

*Synthesis.* Glycine is synthesized by the reaction of chloroacetic acid (1 mole) and ammonia (30 to 60 moles) by a combination of the Robert-

<sup>18, 19</sup> see p. 359.



son (671) and Orten and Hill (600, 601) modifications of the method originated by Perkin and Duppa (631) and Cahours (136). After the mixture has stood overnight at room temperature, the excess ammonia is removed by distillation, 5 volumes of methanol are added to the residual solution, and the resulting glycine is recrystallized from aqueous methanol. A 60% yield of purified glycine is readily obtained. Tobie and Ayres (785) reported that the yield could be increased to 75% of the theoretical amount by distilling the ammoniacal solution to dryness, removing the  $\text{NH}_4\text{Cl}$  from the residue by extraction with methanol, and recrystallizing the crude glycine from aqueous methanol. It was pointed out by Kraut (482, 483) that a relatively high molal ratio of ammonia to chloroacetic acid is required to depress the formation of the side reaction products, diglycolamic and triglycolamic acids, first observed by Heintz (387-390). Chadwick and Pacsu (146) have shown that these secondary reactions are not important with  $\alpha$ -halogen acids other than chloro- or bromoacetic acid.

Comparable yields of glycine were obtained by Cheronis and Spitzmueller (149) in the reaction at 60 to 65°C. of chloroacetic acid and a saturated solution of ammonium carbonate in aqueous ammonia. It was proposed (149, 204) that the proportion of di and tri acids was depressed in this mixture (containing relatively few moles of ammonia per mole of chloroacetic acid) because of the increase in carbaminoglycinate ion resulting from an effect of the carbonate ion. It has been found that glycine may be synthesized satisfactorily with mixtures in which the equivalent ratio of ammonia to carbonate to chloroacetic acid is 5 to 4 to 1.

Glycine is synthesized in about 46% over-all yield from formaldehyde. Methyleneaminoacetonitrile is prepared in about 70% yield by stirring for 6 hours a mixture containing formaldehyde,  $\text{NH}_4\text{Cl}$ , NaCN and glacial acetic acid (43). Aminoacetonitrile hydrogen sulfate is prepared in about 75% yield by shaking a mixture of methyleneaminoacetonitrile,  $\text{H}_2\text{SO}_4$  and ethanol (58, 59). Glycine is prepared by refluxing a mixture of aminoacetonitrile hydrogen sulfate and  $\text{Ba}(\text{OH})_2$  until no more ammonia is evolved, removing the barium ions as  $\text{BaSO}_4$  and recrystallizing the crude glycine obtained from the concentrated filtrate (58, 59). Modifications of these procedures, first studied by Eschweiler (261), and Jay and Curtius (416), have been suggested by other workers (159, 162, 262, 333, 428, 519, 712). Syntheses of glycine from ethyl chloroacetate and potassium phthalimide (337, 346), ethyl cyanoacetate through the intermediate hydrazide and azide (676), 1-amino-2-ethanol (98, 179), aminomethanesulfonic acid and NaCN (53) and nitroacetic acid (171) have been reported.

*Isolation.* Glycine was first isolated from gelatin by Braconnot (121)

in 1820. Since gelatin (183) and silk fibroin (92) contain about 25 and 44%, respectively, of glycine they are the best sources of this amino acid. By Fischer's (284, 285) ester method, the filtrate from glutamic acid hydrochloride suspension is evaporated, the sirup is esterified with absolute ethanol and dry HCl gas, the solution is evaporated and allowed to stand at a low temperature and the resulting hydrochloride of ethyl glycinate is removed and recrystallized from absolute ethanol. No practicable procedure has been described for the hydrolysis of ethyl glycinate and the preparation of glycine since the ester readily condenses to form diketopiperazine and glycine polypeptides. Glycine has been isolated as its ester hydrochloride from hair (38, 132), egg proteins (10, 18, 20, 34, 130, 131, 133, 296, 644), whole animals (cat, chick, rabbit) (13, 17), fish scales (21, 35), wool (34), fibrin (35, 251), cottonseed (27, 28), pea seed (608, 613-615), sunflower seed (26), pumpkin seed (8), soybean (606), squash seed (607), Brazil nuts (604), kidney bean (603), velvet bean (432), and other protein materials. Procedures for the isolation of glycine as its copper salt (715, 728), nitranilate (545, 796), hydantoin (119), betaine chloride (252), trioxalatochromiate (92, 108), 5-nitronaphthalene-1-sulfonate (100, 573), and picrate (175, 183, 319, 503, 504) have been reported.

### 8. Histidine

*Synthesis.* DL-Histidine is synthesized from fructose (or sucrose) in about 15% over-all yield through the reaction of 4-chloromethylimidazole hydrochloride with ethyl acetamidomalonate (46) or ethyl acetamidocyanoacetate (50), by modifications of Pyman's (649) method. 4-Hydroxymethylimidazole picrate is readily prepared in about 55% yield by heating a mixture of ammonia, basic cupric carbonate, formaldehyde and fructose (or acid hydrolyzed sucrose), removing the copper with H<sub>2</sub>S, adding picric acid to the filtrate and recrystallizing the product from water by a modification (191, 794) of the method originated by Parrod (624) and further studied by Weidenhagen *et al.* (851). 4-Hydroxymethylimidazole hydrochloride is prepared by extracting the acidified solution of the picrate with benzene by the method of Pyman (648), or Koessler and Hanke (474). 4-Chloromethylimidazole hydrochloride is prepared in about 85% yield by the reaction of PCl<sub>5</sub> and 4-hydroxymethylimidazole hydrochloride by the method of Pyman (648). Ethyl-2-acetamido-2-carbethoxy-3-imidazolepropionate (A) is prepared in 67% yield by condensing ethyl acetamidomalonate<sup>17</sup> and 4-chloromethylimidazole hydrochloride (B) by the method of Albertson and Archer (46), or  $\alpha$ -acetamido- $\alpha$ -cyano- $\beta$ -imidazolepropionate (C) is prepared in 66% yield by condensing (B) and ethyl acetamidocyanoacetate<sup>20</sup> by the

<sup>17</sup> see p. 359; <sup>20</sup> see p. 360.



method of Albertson and Tullar (50). (A) or (C) is hydrolyzed with  $H_2SO_4$  and the resulting DL-histidine, recrystallized from aqueous ethanol, is obtained in 45 and 61% yields, respectively.

Pyman (649, 650) synthesized DL-histidine monohydrochloride in 23% over-all yield, calculated from diaminoacetone dihydrochloride, by the malonic ester reaction with 4-chloromethylimidazole monohydrochloride and DL-histidine picrate (191, 794) in 11% over-all yield, calculated from 4-hydroxymethylimidazole through the azlactone of glyoxaline-4-formaldehyde. The intermediates required for these syntheses were prepared in relatively high yield by the reactions, (A) citric acid  $\rightarrow$  acetone-dicarboxylic acid (42, 418, 474, 629)  $\rightarrow$  diisonitrosoacetone (474, 628)  $\rightarrow$  diaminoacetone hydrochloride (338, 438, 474)  $\rightarrow$  2-thiol-4-aminomethylimidazole  $\rightarrow$  4-hydroxymethylimidazole  $\rightarrow$  glyoxaline-formaldehyde. The synthesis of 4-hydroxymethylimidazole through the intermediates  $\omega$ -bromoacetylphthalimide,  $\omega$ -acetoxyacetylphthalimide, hydroxyaminoacetone, and 4-hydroxymethyl-2-thiolimidazole has been reported by Jackson and Marvel (415).

DL-Histidine is prepared by racemization most conveniently by heating L-histidine hydrochloride in water for 3 hours at 160-165°C. and removing the HCl with silver hydroxide according to the method of Duschinsky (228, 793). L-Histidine has been racemized by heating it with 20% HCl at 160°C. (331), for 20 hours at 150-160° with excess  $Ba(OH)_2$  (36, 238), with glacial acetic acid and acetic anhydride (96, 582) or acetyl chloride (852), and with NaOH and acetic anhydride (836). According to Jackson and Cahill (414), Neuberger (582) failed to effect racemization of L-histidine with ketene because the latter may not have generated acetic acid in excess of the alkali present.

*Isolation.* L-Histidine is isolated from hemoglobin by the method of Vickery (819). A mixture containing 5 cc. of about 7 N HCl per gram of technical hemoglobin is refluxed 24 hours, the solution is distilled *in vacuo* to remove excess HCl, 0.45 g. of 3,4-dichlorobenzenesulfonic acid per gram of technical hemoglobin is added, the solution is seeded with L-histidine bis 3,4-dichlorobenzenesulfonate, the resulting suspension of the latter compound is filtered, and the product is recrystallized from 5 to 10 times its weight of boiling distilled water. The smaller ratio is employed if the crystals are largely stout prisms (L-histidine disulfonate) and the larger ratio is used if masses of fine needle crystals (L-leucine monosulfonate) are observed. The histidine sulfonate is dissolved in water,  $Ba(OH)_2$  is added to pH 7.2, the suspension of barium 3,4-dichlorobenzenesulfonate dihydrate is filtered, the filtrate is concentrated *in vacuo* and the resulting crystals of L-histidine are recrystallized from about

50% ethanol. The yield of L-histidine is about 6% from purified hemoglobin.

L-Histidine is isolated from dried blood corpuscle paste by Foster and Shemin's (325) modification of the Kossel (476) procedure. The HCl hydrolyzate of corpuscle paste is brought to pH 4.4, the crude precipitate containing tyrosine and leucine is removed, an alcoholic solution of  $\text{HgCl}_2$  is added, the solution is brought to pH 7.5, and the resulting histidine- $\text{HgCl}_2$  suspension is filtered. The complex is decomposed with  $\text{H}_2\text{S}$ , and the resulting histidine monohydrochloride is recrystallized from aqueous ethanol. The yield is about 6%. L-Histidine has been isolated essentially by this procedure from protamines (476, 477), blood proteins (1, 23, 36, 177, 192, 238, 325, 330, 344, 368, 430, 461, 492, 493, 627, 665), seeds and germinated seedlings (696, 697, 703, 705, 707), egg proteins (644, 730), fish proteins (475, 770), and other materials. Salts in addition to those mentioned which have been utilized for the isolation of histidine from various proteins include the silver salt (480), the phosphotungstate and the silver salt (383, 489, 729, 772), the silver salt and the  $\text{HgSO}_4$  complex (481, 605, 617, 698, 824, 825, 830), the silver salt, the  $\text{HgSO}_4$  complex and the diflavinate (89, 90, 104-106, 112, 818, 820, 823, 880), the phosphotungstate or  $\text{HgSO}_4$  complex, the silver salt and the picolonate (63, 129, 441, 456, 527, 566, 764, 774), and the nitranilate (107, 109). The use of dioxane and azeotropic mixtures of dioxane, isopropanol and *n*-propanol for the extraction of histidine and other amino acids has been suggested by Gilson (344, 345). L-Histidine is prepared by dissolving L-histidine mono- or dihydrochloride in water, adding an excess of hot concentrated  $\text{Ag}_2\text{SO}_4$  (476, 821), filtering the suspension, removing the silver ions from the filtrate as silver sulfide and adding an equal volume of ethanol to the filtrate. The free base may be prepared as readily by adding an equivalent quantity of lithium hydroxide (230) or concentrated ammonia and an equal volume of ethanol or methanol to a water solution of L-histidine hydrochloride.

### 9. Hydroxyproline

*Synthesis.* Hydroxyproline is synthesized by the method of McIlwain and Richardson (542).  $\alpha$ -Acetyl- $\delta$ -chloro- $\gamma$ -valerolactone, prepared from acetoacetic ester and epichlorohydrin<sup>21</sup> by the method of Leuchs (497), is converted to  $\alpha$ -oximino- $\delta$ -chloro- $\gamma$ -valerolactone (A) by the action of nitrosyl sulfuric acid. (A) is reduced to  $\alpha$ -amino- $\delta$ -chloro- $\gamma$ -valerolactone (B) by shaking it for 3 days with hydrogen and platinum oxide catalyst. (B) is converted to a mixture of the isomeric DL-hydroxyprolines by treatment for 2 hours with a saturated aqueous solution of ammonia. The copper salts of the two pairs of optical isomers are pre-

<sup>21</sup> see p. 360.

pared and fractionally crystallized from water yielding a slightly soluble copper salt of DL-hydroxyproline and an easily soluble copper salt of DL-allo-hydroxyproline. The DL-hydroxyprolines are prepared by removing the copper with  $\text{H}_2\text{S}$  and crystallizing the amino acids from aqueous-ethanol. According to the evidence of Leuchs (496), and Leuchs and coworkers (499, 500, 502), L-hydroxyproline, prepared by resolution of DL-hydroxyproline is probably identical with (natural) L-hydroxyproline. Essentially the same synthesis has been reported by Feofilaktov and Onishchenko (267, 269) who reduced the  $\alpha$ -oximino derivative and the  $\alpha$ -phenylhydrazone of  $\delta$ -chloro- $\gamma$ -valerolactone with zinc and HCl.

The DL-hydroxyprolines, synthesized and resolved by Leuchs and coworkers (496, 498-500, 502) were prepared by the reactions, malonic ester + epichlorhydrin  $\rightarrow$  ethyl  $\delta$ -chloro- $\gamma$ -valerolactone- $\alpha$ -carboxylate  $\rightarrow$   $\alpha$ - $\delta$ -dichloro- $\gamma$ -valerolactone<sup>22</sup>  $\rightarrow$   $\alpha$ , $\delta$ -diamino- $\gamma$ -valerolactone  $\rightarrow$  hydroxyproline + allo-hydroxyproline. Another synthesis through the intermediates, ethyl- $\gamma$ , $\delta$ -dibromo- $\alpha$ -benzoylamino-*n*-valerate and  $\gamma$ -(benzoylhydroxy) proline, has been reported by Hammarsten (367).

Natural hydroxyproline is racemized by heating with  $\text{Ba}(\text{OH})_2$  or glacial acetic acid and acetyl chloride only on the  $\alpha$ -carbon atom according to Leuchs and coworkers (498-500, 502) and Werner (852).

*Isolation.* L-Hydroxyproline is isolated in 6.5% yield from gelatin by the method of Neuberg (583). Gelatin is hydrolyzed with HCl, the arginine is removed as the flavianate, lysine and histidine are removed from the diluted filtrate as their phosphotungstates, excess phosphotungstic and flavianic acids in the filtrate are extracted with a mixture of aqueous ammonia and ether, proline and hydroxyproline are precipitated as their reineckates, the latter are decomposed with  $\text{CuSO}_4$  and  $\text{SO}_2$ , copper is removed as  $\text{Cu}_2\text{S}$ , sulfate is removed as  $\text{BaSO}_4$ , and the residual solid is extracted with absolute ethanol to remove proline. The crude hydroxyproline is recrystallized from about 80% ethanol. The specific rotation in water,  $[\alpha]_D = -76.0^\circ$  to  $-76.5^\circ$ , is in reasonable agreement with the value,  $[\alpha]_D^{26} = -75.7^\circ$  reported by Leuchs and Bormann (498) and the value,  $[\alpha]_D^{20} = -75.2^\circ$  (unpublished data by M. P. Stoddard in the writers' laboratory).

L-Hydroxyproline has been isolated from gelatin, hemoglobin and other proteins by other procedures but few, if any, of the products were proved to be pure. L-Hydroxyproline was first isolated by Fischer (281) by crystallization from a gelatin hydrolyzate from which glycine had been removed as its ethyl ester hydrochloride and the diamino acids as their phosphotungstates. Other workers (1, 3, 183, 729) have followed this procedure. Methods have been described for the isolation of hydroxyproline by means of its picrate (466), cadmium chloride complex (441),

<sup>22</sup> see p. 360.

platinum and gold chloride complexes (252), and N-acetyl-O-benzoyl derivative (771). Kapfhammer and Eck (440) isolated 7% of hydroxyproline from a gelatin hydrolyzate from which arginine had been removed as the flavanate, and proline and hydroxyproline as their reineckates. The latter were decomposed by treatment with  $\text{CuSO}_4$  and  $\text{SO}_2$ . Hydroxyproline was separated from proline and purified as described by Neuberger (583). Bergmann (85) isolated 10.5% of hydroxyproline from a gelatin hydrolyzate from which arginine had been removed as the flavanate, proline as the rhodanilate, and hydroxyproline as the reineckate. The latter was decomposed by treatment with pyridine.

### 10. Isoleucine

*Synthesis.* DL-Isoleucine is synthesized in about 27% over-all yield by the method of Marvel (554). Diethyl sec.-butylmalonate (A) is prepared in 83% yield from diethyl malonate, sodium, absolute ethanol and sec.-butyl bromide (b.p.  $91.3^\circ\text{C}$ .) essentially by the method of Romburgh (673).  $\alpha$ -Bromo- $\beta$ -methylvaleric acid<sup>23</sup> (B) is prepared in about 67% yield by the alkaline hydrolysis of (A), isolation of sec.-butylmalonic acid (C), and bromination and decarboxylation of (C). DL-Isoleucine (D), prepared by amination of (B), is recrystallized from 30% ethanol. It has been suggested (39, 485) that the product should be repeatedly recrystallized from 80% ethanol to free it from allo-isoleucine. It has been found in the writers' laboratory that recrystallization from 20% ethanol is an effective purification procedure. The described synthesis is essentially that originated by Brasch and Friedman (125) and Ehrlich (237) and employed by Abderhalden and Zeisset (39).

DL-Isoleucine is synthesized in about 56% over-all yield by the method of Hamlin and Hartung (366).  $\alpha$ -Oximino- $\beta$ -methyl-*n*-valeric acid (A) is prepared in 70% yield from ethyl sec.-butyl acetoacetate, butyl nitrite and sulfuric acid. DL-Isoleucine is prepared in 80% yield by the reduction of (A) with hydrogen, palladium chloride and ethanol. This method is essentially the same as that originated by Bouveault and Locquin (117, 118, 525). By the comparable procedure of Feofilaktov (264, 265) the phenylhydrazone of methyl ethyl pyruvic acid, prepared from sec.-butyl acetoacetate and phenyldiazonium chloride (aniline and  $\text{NaNO}_2$ ) in 68% yield, is reduced by means of zinc and alcoholic HCl to nearly the theoretical yield of a mixture of DL-isoleucine and DL-allo-isoleucine. Ehrlich (235) synthesized a mixture of isoleucine and allo-isoleucine from 2-methyl-*n*-butyraldehyde by the Strecker reaction.

Natural isoleucine is racemized by heating it with  $\text{Ba}(\text{OH})_2$  for 20 hours at  $180^\circ\text{C}$ . (233) or with glacial acetic acid and acetyl chloride for 1 hour at  $100^\circ\text{C}$ . (852). It is only partly racemized by treatment with

<sup>23</sup> see p. 360.

ketene and alkali (134). No racemization is effected by refluxing it for 18 hours with 20% HCl (511).

*Isolation.* L-Isoleucine was isolated from de-sugared molasses by Ehrlich (233) who separated the less soluble copper salt of L-leucine from the more soluble copper salt of L-isoleucine by fractional crystallization from methanol. This procedure has been employed for the isolation of isoleucine from lupine seed (873), silkworm egg cases (790), brain (7, 720), fibrin (91), mold (881), and other proteins but there is no practicable procedure for the isolation of analytically pure L-isoleucine. According to Locquin (524) the specific rotation of pure resolved L-isoleucine in 20% HCl is  $[\alpha]_D^{20} = +40.6^\circ$ . Approximately the same value,  $[\alpha]_D^{20} = +40.0^\circ$ , was found by Bauer and Berg (7) under the same conditions. Isoleucine has been isolated from cod muscle protein (119) as the  $\alpha$ -carbamido and the hydantoin derivatives and from crude commercial DL-Leucine (385) as the *p*-toluenesulfonyl derivative.

### 11. Leucine

*Synthesis.* DL-Leucine is synthesized in 29% over-all yield by Marvel's (555) modification of the method of Fischer (282). DL- $\alpha$ -Bromoisocaproic acid<sup>24</sup> is prepared in 65% yield by refluxing isocaproic acid,<sup>25</sup> PCl<sub>3</sub> and bromine in dry benzene for 8-15 hours. DL-Leucine is prepared in 44% yield by the reaction of 55 moles of aqueous ammonia per mole of DL- $\alpha$ -bromoisocaproic acid and recrystallization of the crude product from 50% ethanol.

DL-Leucine is synthesized in 28% over-all yield by the method of Redemann and Dunn (660) from diethyl malonate through the intermediates, diethyl isonitrosomaltonate, diethyl aminomaltonate, diethyl benzamidomaltonate and diethyl- $\alpha$ -isobutyl- $\alpha$ -benzamidomaltonate<sup>26</sup>. It is synthesized from diethyl acetamidomaltonate<sup>17</sup> in 70% over-all yield by the method of Albertson and Archer (46) through the intermediates, ethyl methyl allylacetamidomaltonate and ethyl isobutylacetamidomaltonate. DL-N-Acetylleucine has been prepared in 29% yield by Snyder *et al.* (740) from diethyl acetamidomaltonate<sup>17</sup> through the intermediate, diethyl isobutylacetamidomaltonate. DL-Leucine has been prepared in 50% yield by Albertson and Tullar (50) from ethyl acetamidocyanoacetate<sup>20</sup> through the intermediate, ethyl isobutylacetamidocyanoacetate.

Syntheses of leucine from isovaleraldehyde by the Strecker reaction (704), ethyl isobutylacetoacetate by reduction of the intermediates  $\alpha$ -oximinoisocaproic acid (116) and the phenylhydrazone of isopropylpyruvic acid (264-267), isobutylhydantoin by alkaline hydrolysis (633), isobutyraldehyde by the azlactone method (259), and ethyl cyanoacetate

<sup>17</sup> see p. 359; <sup>20</sup>, <sup>24-26</sup> see p. 360.

through the intermediates, ethyl isobutyl cyanoacetate and the hydrazide and azide of isobutylcyanoacetic acid (190) have been reported.

DL-Leucine and some of its derivatives have been prepared by heating the active form with PbO for 7 hours at 165°C. (278), Ba(OH)<sub>2</sub> for 3 days at 150-160°C. (702) or 24 hours at 170°C. (285), acetic anhydride and NaOH (279), acetyl chloride and glacial acetic acid (852), PCl<sub>5</sub> (444), and ketene and NaOH (135).

*Isolation.* L-Leucine is isolated from hemoglobin by the method of Stein *et al.* (755). Technical hemoglobin is hydrolyzed with sulfuric acid, an equivalent amount of Ba(OH)<sub>2</sub> is added, the suspension of BaSO<sub>4</sub> is filtered, the filtrate and washings are concentrated *in vacuo*, the residual liquid is allowed to stand overnight at 0°C., the suspension is filtered and the precipitate is dried. The dry powder is dissolved in boiling water, the solution is decolorized with acid-washed carbon, 12 N HCl is added to the hot filtrate, and an aqueous solution of sodium 2-bromotoluene-5-sulfonate is added to the acid mixture. The resulting leucine salt is recrystallized from water, BaCl<sub>2</sub>·2H<sub>2</sub>O is added to a solution of the product, the suspension of barium 2-bromotoluene-5-sulfonate is filtered, and the crude leucine obtained from the filtrate is recrystallized by dissolving it in ammonium hydroxide and evaporating the ammonia. The yield of sulfur-free L-leucine,  $[\alpha]_D^{26} = +15.5^\circ$  in 21% HCl, is about 8%.

In 1820, Braconnot (121) hydrolyzed wool with sulfuric acid, added calcium carbonate, evaporated the filtrate and crystallized leucine from the sirup. Essentially this procedure has been employed by most workers since that time, although it has been common practice to remove tyrosine, glutamic acid, glycine and proline from hydrolyzates and to obtain a crude leucine fraction by fractional distillation of the ethyl esters or by fractional crystallization of the copper or other metal salts of the remaining amino acids. A crude precipitate containing leucine as well as tyrosine, methionine (580) and other amino acids has been obtained at pH 4 in some procedures as a preliminary step in the isolation of the basic amino acids. Measures taken to purify the crude leucine include extraction with hot glacial acetic acid<sup>27</sup> to eliminate the slightly soluble tyrosine (361), removal of valine as its water-soluble lead salt (511) and removal of isoleucine as its hot methanol-soluble copper salt (508). It has been found more recently that L-leucine, free from methionine and apparently from other amino acids, is readily prepared by fractionally crystallizing crude leucine<sup>28</sup> as its formyl (326),  $\beta$ -naphthalenesulfonyl (93) or monohydrochloride (759) derivative. Hotchkiss (409) has shown that D-leucine is present in gramicidin, an antibiotic substance elaborated

<sup>27, 28</sup> see p. 360.



by *Bacillus brevis*, by isolating this amino acid as its  $\beta$ -naphthalene-sulfonate.

## 12. Lysine

*Synthesis.* DL-Lysine is synthesized from cyclohexanone in 22% overall yield by the method of Eck and Marvel (231, 232). A cold solution of sodium bisulfite (solution of anhydrous sodium carbonate saturated with  $\text{SO}_2$ ) is added to a mixture of sodium nitrite and cracked ice kept cold with an ice-salt bath.  $\text{SO}_2$  is passed into the solution until it is acid, cyclohexanone and ethanol are added, the mixture is heated to  $75^\circ\text{C}$ . and cooled slowly, the solution is neutralized with  $\text{NaOH}$ , the oily layer is separated, the aqueous layer is extracted with ether, and the combined oil and ether extract are distilled *in vacuo*. The yield of cyclohexanoneoxime<sup>29</sup> is about 60%. Ten gram portions of the product, cyclohexanoneoxime, and 20 cc. of about 30 N  $\text{H}_2\text{SO}_4$  are heated gently in separate flasks<sup>30</sup> until bubbles appear, the flasks are set aside until the initial violent reaction subsides, the acid solutions from the separate flasks are combined, and the mixture is diluted and refluxed for 1.5 hours to hydrolyze the lactam (2-ketohexamethylenimine). The solution is filtered and made alkaline, benzoyl chloride is added dropwise, the mixture is acidified, and the resulting suspension is filtered. The yield of  $\epsilon$ -benzoylaminocaproic acid is about 70%. A cold mixture of this product and dry red phosphorus is stirred, dry bromine is added dropwise, the excess bromine is removed by distillation and by treatment with  $\text{SO}_2$ , and the solid product is washed and dried. The yield of  $\alpha$ -bromo- $\epsilon$ -benzoylaminocaproic acid is about 90%. A mixture of this bromoacid and excess ammonia is allowed to stand for 2 days at room temperature, the solution is evaporated *in vacuo*, and the resulting suspension is filtered and washed. The yield of  $\epsilon$ -benzoyllysine is about 70%. This product is refluxed for 10 hours with  $\text{HCl}$ , the suspension of benzoic acid is filtered, the filtrate is evaporated, absolute ethanol and ether are added to the sirup, and the suspension is filtered. The yield of nearly pure lysine dihydrochloride is about 85%. DL-Lysine monohydrochloride is readily prepared by crystallizing DL-lysine dihydrochloride from a mixture of ethanol and aqueous ammonia.

Syntheses of lysine have been reported from malonic ester through the intermediates,  $\gamma$ -cyanopropylmalonic ester and  $\alpha$ -oximido- $\delta$ -cyanovaleric acid ester (311), from phthalimidomalonic ester through phthalimidobutyl phthalimidomalonic ester (744), from benzoylpiperidine (A) by the reactions: (A)  $\rightarrow$  1-chloro-5-benzoylaminopentane  $\rightarrow$  1-cyano-5-benzoylaminopentane  $\rightarrow$   $\epsilon$ -benzoylaminocaproic acid  $\rightarrow$   $\alpha$ -bromo- $\epsilon$ -benzoylaminocaproic acid (126, 557), from acrolein through a series of

<sup>29, 30</sup> see p. 360.

intermediates including  $\epsilon$ -benzoylaminocaproic acid (768), and from  $\alpha$ -aminopimelic acid or ethyl cyclohexanone-2-carboxylate and hydrazoic acid (44).

The active form of lysine has been racemized with  $\text{Ba}(\text{OH})_2$  (722, 723),  $\text{HCl}$  (477), acetyl chloride and glacial acetic acid (852) and ketene and  $\text{NaOH}$  (582).

*Isolation.* L-Lysine is isolated as the monohydrochloride from blood corpuscle paste by the method of Rice (665). Blood corpuscle paste is hydrolyzed with sulfuric acid, the sulfate ions are removed with  $\text{Ba}(\text{OH})_2$  or  $\text{Ca}(\text{OH})_2$ , the filtrate is concentrated *in vacuo*, the suspension of amino acids is filtered, picric acid is added to the filtrate, and the mixture is heated until the solid dissolves and the mixture is allowed to stand overnight in a refrigerator. The resulting suspension of lysine picrate is filtered, the precipitate is washed and dissolved in water, the solution is decolorized with norite, and lysine picrate is allowed to crystallize from the filtrate. This product is dried in air and heated with 6 N  $\text{HCl}$ , the precipitated picric acid is removed, the filtrate is extracted with hot benzene, the aqueous solution is decolorized with norite, the filtrate is concentrated, the sirup is dissolved in ethanol, ether is added, and the resulting suspension of lysine dihydrochloride is filtered. The product is dried and recrystallized from a mixture of ethanol and ether, the purified dihydrochloride is dissolved in ethanol, pyridine is added, and the resulting suspension of lysine monohydrochloride is filtered. The yield of nearly pure L-lysine monohydrochloride, once recrystallized from aqueous ethanol, is about 12.5 g. (1.25%) per kg. of blood corpuscle paste.

Cox, King and Berg (177) prepared lysine in about the same yield essentially by the procedure of Rice, except that arginine and lysine were separated by electrodialysis and arginine was removed from the catholyte by precipitation as the flavianate before crystallizing the lysine picrate. Kossel (477) first isolated lysine as the picrate after removing histidine as the  $\text{HgCl}_2$  complex and arginine as the silver salt. By a later procedure (478), the basic amino acids were precipitated with phosphotungstic acid according to the method of Drechsel (200, 201), and histidine and arginine were separated from lysine as their silver salts before precipitating lysine picrate. This method and later modifications by Kossel and coworkers have been used widely by Schulze, Abderhalden, Skraup, Meisenheimer, Osborne, Vickery, Jones, Calvery, Block and other workers primarily for the determination of lysine in proteins.<sup>31</sup> The isolation of lysine as the silver nitrate double salt (200), the dibenzoyl derivative (201), the chloroplatinate (199), the benzyldiene

<sup>31</sup> see p. 360.



and O-hydroxybenzylidene derivatives (95) and  $\epsilon$ -benzoyllysine copper (487) has been reported.

### 13. Methionine

*Synthesis.* DL-Methionine is synthesized by the following methods.

*Method One*, Goldsmith, and Tishler (347). Diethyl acetamido-(2-methylmercaptoethyl)-malonate (A) is prepared as a crude solid from sodium, diethylacetamidomalonate<sup>17</sup> and 2-methylmercaptoethyl chloride<sup>32</sup> in tert.-butyl alcohol by refluxing the mixture for 6 hours. (A) is refluxed for 9 hours with 12 N HCl, the mixture is evaporated to dryness, the residue is dissolved in ethanol, pyridine is added, and the resulting DL-methionine is recrystallized from 50% ethanol. The yield of nearly pure product is about 60%.

*Method Two*, Albertson and Tullar (50).  $\beta$ -methylthioethanol is prepared in 80% yield from monothioethylene glycol and dimethylsulfate. This product is chlorinated by action of thionyl chloride or HCl, and the intermediate,  $\beta$ -methylthioethyl chloride, is condensed in solution with ethyl acetamidocyanoacetate<sup>20</sup> to give a 60% yield of ethyl  $\alpha$ -acetamido- $\alpha$ -cyano- $\gamma$ -methylthiolbutyrate. The latter is hydrolyzed with base to give 80% yield of recrystallized DL-methionine. The over-all yield of product is about 48%.

*Method Three*, Livak *et al.* (521).  $\alpha$ -Bromo- $\gamma$ -butyrolactone (A) is prepared in 80% yield from  $\gamma$ -butyrolactone (commercially available), PBr<sub>3</sub> and bromine.  $\alpha$ -Amino- $\gamma$ -hydroxybutyric acid (B) is prepared in 60% yield by amination of (A). 5-( $\beta$ -Bromoethyl)-hydantoin (C) is prepared in 51% yield by the reaction of (B) with potassium cyanate to give  $\gamma$ -hydroxy- $\alpha$ -ureidobutyric acid (D) and by the HBr hydrolysis of (D). 5-( $\beta$ -Methylmercaptoethyl)-hydantoin (E) is prepared in 73% yield from sodium methylmercaptide<sup>25</sup> and (C). DL-Methionine is prepared in 95% yield by the Ba(OH)<sub>2</sub> hydrolysis of (E). The over-all yield is about 17%.

*Method Four*, Snyder *et al.* (736).  $\alpha$ -Oximino- $\gamma$ -butyrolactone (A) is prepared in 90% yield from acetobutyro- $\gamma$ -lactone<sup>33</sup> and ethyl nitrite in methanol. 3,6-Bis-( $\beta$ -hydroxyethyl)-2,5-diketopiperazine<sup>34</sup> (B) is prepared in 60% yield from (A) through  $\alpha$ -amino- $\gamma$ -butyrolactone by reduction of the oxime with hydrogen and a palladium catalyst. 3,6-Bis-( $\beta$ -chloroethyl)-2,5-diketopiperazine (C) is prepared in 90% yield from (B) and thionyl chloride. 3,6-Bis-( $\beta$ -methylthioethyl)-2,5-diketopiperazine (D) is prepared in 60% yield from (C) and sodium methylmercaptide<sup>32</sup>. DL-Methionine is prepared in 90% yield by the HCl hydrolysis of (D). The over-all yield is 26%.

*Method Five*, Hill and Robson (400). Ethyl- $\alpha$ -benzamido- $\gamma$ -chloro-

<sup>17</sup> see p. 359; <sup>20-24</sup> see p. 360.

butyrate<sup>35</sup> (A) is prepared in 78% yield from  $\alpha$ -benzamido- $\gamma$ -butyrolactone and HCl. Benzoylmethionine (B) is prepared in 72% yield from (A) and sodium methylmercaptide<sup>32</sup> by alkaline hydrolysis of the resulting benzoylmethionine ethyl ester. Methionine is prepared in 55% yield by acid hydrolysis of (B). The over-all yield is 31%.

*Method Six*, Snyder and Cannon (737). 3,6-Bis-( $\beta$ -isothiuronium-ethyl)-2,5-diketopiperazine chloride (A) is prepared in 98% yield from 3,6-bis-( $\beta$ -chloroethyl)-2,5-diketopiperazine (736) and thiourea. Methionine anhydride<sup>36</sup> (B) is prepared in 75% yield from (A), dimethyl sulfate and NaOH. Methionine is prepared in 65% yield by hydrolysis of (B). The over-all yield is 49%.

*Method Seven*, Barger and Coyne (65). Methionine is prepared in 3% over-all yield from  $\beta$ -chloropropaldehyde acetal by the Strecker reaction of the intermediate  $\beta$ -methylthiolpropaldehyde.

*Method Eight*, Windus and Marvel (866), and modifications by Emerson *et al.* (249). Methionine is prepared in 8% over-all yield from  $\beta$ -chloromethylethylsulfide by the malonic ester reaction.

*Method Nine*, Booth *et al.* (114). Methionine is prepared in 40% over-all yield from  $\beta$ -chloroethylmethyl sulfide by a modification of the phthalimido-malonic ester reaction of Barger and Weichselbaum (67, 68). According to Snyder *et al.* (739), 5% of pseudomethionine (two molecules of methionine joined by the loss of two hydrogen atoms) is present in methionine prepared by large-scale adaptation of the Barger-Weichselbaum synthesis.

DL-Methionine was isolated in 1923 by Mueller (579) from casein which had been autoclaved for 14 hours with 18% NaOH. Methionine was racemized by du Vigneaud and Meyer (840) by heating the natural form with acetic anhydride in *N* NaOH for 5 hours at 37°C.

*Isolation.* L-Methionine has been isolated from casein and other proteins by Mueller (15, 399, 577-579, 634, 635) and other workers (65, 594, 773) by a mercury salt procedure<sup>37</sup> but only small amounts of the amino acid were obtained and most of the products were somewhat impure. The specific rotation in water,  $-7.3^\circ$ , was obtained by du Vigneaud and Meyer (839) at 25°C. Values at 25°C. for the specific rotation of L-methionine reported by Windus and Marvel (867) were  $-6.9^\circ$  in water (natural form isolated from casein) and  $-7.5^\circ$  in water (resolved product).

#### 14. Phenylalanine

*Synthesis.* DL-Phenylalanine is synthesized by the following methods.

*Method One*, Marvel's (556) modification of Fischer's (283) procedure. Diethyl benzylmalonate<sup>38</sup> is prepared in 55% yield from sodium, absolute

<sup>32-37</sup> see p. 360; <sup>28</sup> see p. 361.

ethanol, diethyl malonate and benzyl chloride.  $\alpha$ -Bromo- $\beta$ -phenylpropionic acid is prepared as a crude oil by alkaline hydrolysis of diethyl benzylmalonate and bromination and decarboxylation of the resulting benzylmalonic acid. DL-Phenylalanine is prepared in 60% yield (based on diethyl benzylmalonate) by amination of  $\alpha$ -bromo- $\beta$ -phenylpropionic acid and recrystallization of the crude product from about 25% ethanol. The over-all yield is 34%.

*Method Two*, Gillespie and Snyder's (343) modification of the Plöchl-Erlenmeyer (253, 639, 640) method. The azlactone of  $\alpha$ -benzoylamino-cinnamic acid (A) is prepared in 63% yield from benzaldehyde, hippuric acid, sodium acetate and acetic anhydride. DL-Phenylalanine is prepared in 65% yield by reducing (A) with HI and red phosphorus<sup>39</sup> in acetic anhydride. The over-all yield is 41%. The crude product may be recrystallized by dissolving it in boiling water and evaporating the solution.

*Method Three*, Herbst and Shemin (394). Acetylglucine is prepared in 90% yield from glycine and acetic anhydride essentially by the method of Radenhausen (652).  $\alpha$ -Acetaminocinnamic acid is prepared in 64% yield from acetylglucine, sodium acetate, benzaldehyde and acetic anhydride by isolating the intermediate azlactone and refluxing it with aqueous acetone. This procedure is essentially the same as that of Erlenmeyer and Früstück (256), and Bergmann and Stern (94). DL-Phenylalanine is prepared in 85% yield by reduction of  $\alpha$ -acetaminocinnamic acid in glacial acetic acid with hydrogen and platinum oxide and HCl hydrolysis of the resulting N-acetylphenylalanine. The crude DL-phenylalanine is recrystallized from 65% ethanol. The over-all yield is 65%.

*Method Four*, Hamlin and Hartung (366, 849).  $\alpha$ -Oximino- $\beta$ -phenylpropionic acid (A) is prepared in 90% yield from benzylacetoacetate, butyl nitrite and sulfuric acid. DL-phenylalanine is prepared in 89% yield by the reduction of (A) with hydrogen, palladium chloride and ethanol. The over-all yield is 79%.

*Method Five*, Albertson and Archer (46). Ethyl 2-acetamido-2-carbethoxy-3-phenylpropionate (A) is prepared in 90% yield from benzyl chloride and ethyl acetamidomalonate<sup>17</sup>. DL-Phenylalanine<sup>40</sup> is prepared in 65% yield by HBr or NaOH-HCl hydrolysis of (A). The over-all yield is 58%. (Comparable syntheses of phenylalanine from benzyl chloride and diethyl aminomalonate (144, 526) or diethyl benzamidomalonate (660) have been reported).

*Method Six*, Albertson and Tullar (50). Ethyl- $\alpha$ -acetamido- $\alpha$ -cyano- $\beta$ -phenylpropionate (A) is prepared in 83% yield from benzyl chloride and ethyl acetamidocyanoacetate<sup>20</sup>. DL-Phenylalanine is prepared in

<sup>17</sup>, <sup>20</sup> see p. 359, 360; <sup>39</sup>, <sup>40</sup> see p. 361.

75% yield by alkaline hydrolysis of (A). The over-all yield is 62%.

*Method Seven*, Sasaki (677). 3,6-Dibenzal-2,5-diketopiperazine (A) is prepared in 62% yield from benzaldehyde, sodium acetate, acetic anhydride and 2,5-diketopiperazine<sup>41</sup>. DL-Phenylalanine is prepared in 83% yield by reduction of (A) to the intermediate 3,6-dibenzyl-2,5-diketopiperazine (B) with HI and red phosphorus and simultaneous hydrolysis of (B). The over-all yield is 50%.

*Method Eight*, Sørensen and Anderson (748). Diethyl benzylphthalimidomalonate (A) is prepared in 80% yield from benzyl chloride and sodium diethyl phthalimidomalonate. DL-Phenylalanine is prepared in 80% yield by hydrolysis of (A). The over-all yield is 64%.

Syntheses of phenylalanine by reducing the oxime of phenylpyruvic acid (351, 469, 642, 718), catalytically reducing and aminating phenylpyruvic acid (470, 471), hydrolyzing  $\alpha$ -cyano- $\beta$ -phenylpropionazide obtained from ethyl  $\alpha$ -cyano- $\beta$ -phenylpropionate through the intermediate  $\alpha$ -cyano- $\beta$ -phenylpropionhydrazide (339), hydrolyzing 4-benzylhydantoin obtained from hydantoin and benzaldehyde through the intermediate 4-benzalhydantoin (422, 859), hydrolyzing 2-thio-4-benzylhydantoin obtained from 2-thiohydantoin through the intermediate 2-thio-4-benzalhydantoin (425, 427), reducing the phenylhydrazone of phenylpyruvic acid obtained from ethyl benzylacetoacetate and potassium phenyldiazotate (264, 266, 270, 271) and reaction of benzylmalonic acid, hydrazoic acid and sulfuric acid (128) have been reported.

Phenylalanine has been racemized by heating the active form with acetic anhydride and glacial acetic acid (468) or NaOH (840) and with NaOH and ketene (414).

*Isolation*. L-Phenylalanine is isolated from hemoglobin by the method of Stein *et al.* (755). The filtrate (page 315) obtained in the precipitation of L-leucine 2-bromotoluene-5-sulfonate is treated with 2,5-dibromobenzene sulfonic acid, the mixture is allowed to stand overnight at 0°C. and the resulting L-phenylalanine 2,5-dibromobenzenesulfonate is recrystallized from aqueous methyl cellosolve. The crude product is dissolved in a mixture of water and pyridine, hot ethanol is added to the solution, the mixture is allowed to stand overnight at 0°C. and the resulting crude phenylalanine is recrystallized from a mixture of water, ammonia and ethanol. The yield of purified phenylalanine is about 35 g. (1.75%) from 2 kg. of technical hemoglobin. The specific rotation,  $[\alpha]_D^{26} = -34.0^\circ$  (2% solution in water), was found by Stein *et al.* According to Dalton and Schmidt (186),  $[\alpha]_D^{25} = -35.0^\circ$  in water (see Table VI). L-Phenylalanine was first isolated as its copper salt from extracts of lupine seedlings by Schulze and Barbieri (700) in 1881. During the following 20 years, Schulze and coworkers isolated phenylalanine from numerous

<sup>41</sup> see p. 361.

plant species by this procedure. In 1901, Fischer (280) obtained phenylalanine from an acid hydrolyzate of casein by fractionally distilling *in vacuo* the ethyl esters of the amino acids and fractionally crystallizing the amino acids liberated by  $\text{Ba}(\text{OH})_2$  hydrolysis from the ether soluble portion of the highest boiling ester fraction. Although partial or complete racemization resulted from this treatment, essentially this method was applied to numerous proteins by Fischer, Abderhalden, Schulze, Winterstein, Osborne, and most other workers during the succeeding 30 years. Few, if any, of the described procedures have practicable value for isolation purposes.

### 15. Proline

*Synthesis.* Numerous syntheses of proline have been reported but none of the methods appears to be entirely satisfactory as a laboratory procedure. The proline synthesized was of uncertain purity and, even today, the physical properties of analytically pure DL- or L-proline have not been determined. DL-Proline has been synthesized by the following methods.

*Method One*, Willstätter (862-864). Diethyl sodium malonate + trimethylene bromide  $\rightarrow$  diethyl  $\gamma$ -bromopropylmalonate<sup>42</sup>  $\rightarrow$  diethyl bromo- $\gamma$ -bromopropylmalonate  $\rightarrow$  diethyl amino- $\gamma$ -aminopropylmalonate  $\rightarrow$   $\alpha,\alpha$ -pyrrolidinedicarboxylic acid diamide  $\rightarrow$  proline.

*Method Two*, Sørensen (745). Potassium phthalimide + trimethylene bromide + diethyl malonate  $\rightarrow$  diethyl  $\gamma$ -phthalimidopropylmalonate  $\rightarrow$  diethyl bromo- $\gamma$ -phthalimidopropylmalonate  $\rightarrow$  proline.

*Method Three*, Sørensen (313, 748, 749). Sodium diethyl phthalimidomalonate + trimethylene bromide  $\rightarrow$  diethyl phthalimido- $\gamma$ -bromopropylmalonate  $\rightarrow$  proline.

*Method Four*, Fischer and Zemplén (313, 314). Piperidine  $\rightarrow$  N-benzoylpiperidine  $\rightarrow$   $\delta$ -benzoylvaleric acid  $\rightarrow$   $\alpha$ -bromo- $\delta$ -benzoylaminovaleric acid  $\rightarrow$   $\alpha$ -amino- $\delta$ -benzoylaminovaleric acid  $\rightarrow$  proline. The synthesis through the corresponding *m*-nitrobenzoyl derivatives was described by Fischer and Zemplén (314). The preparation of  $\alpha$ -bromo- $\delta$ -benzoylaminovaleric acid (A) from cyclopentanone oxime through the intermediates  $\alpha$ -piperidone,  $\delta$ -aminovaleric acid and  $\delta$ -benzoylaminovaleric acid has been reported by Schniepp and Marvel (688). Mayeda and Nozoe (564) synthesized (A) essentially by the method of Schniepp and Marvel. Copper prolinic acid dihydrate was prepared from (A).

*Method Five*, Heymans (395). The N-benzoyl or N-*m*-nitrobenzoyl derivative of  $\beta,\beta$ -dibromo- $\alpha$ -piperidone, formed as side reaction product in the synthesis of  $\alpha$ -bromo- $\delta$ -benzoylaminovaleric acid, or  $\alpha$ -bromo- $\delta$ -

<sup>42</sup> see p. 361.

*m*-nitrobenzoylaminovaleric acid (Method Four) is converted to  $\alpha,\alpha$ -dibromo- $\delta$ -aminovaleric acid (A). Proline is formed by reducing (A) with Na-Hg.

*Method Six*, Putochin (646). Diethyl aminomalonate + trimethylene bromide  $\rightarrow$  diethyl  $\gamma$ -bromopropylmalonate  $\rightarrow$   $\alpha$ -pyrrolidinedicarboxylic acid  $\rightarrow$  proline.

*Method Seven*, Signaigo and Adkins (725). Pyrrole + ethyl magnesium bromide + ethyl chlorocarbonate  $\rightarrow$  1,2-dicarbethoxypyrrole  $\rightarrow$  1,2-dicarbethoxypyrrolidine  $\rightarrow$  proline. Adkins and coworkers (45, 653, 724) have shown that the pyrrole nucleus requires more drastic conditions for reduction than the benzenoid nucleus, that the N-carbethoxy substituent decreases resonance so that the pyrrole ring may be hydrogenated under mild conditions and that this substituent is more effective in the 1- than the 2-position. The synthesis of proline by the reduction of 2-carboxypyrrole (312, 297, 647) or of 2-carboxy- $\alpha$ -pyrrolidone (294, 539, 540) has not been successful for these reasons.

*Method Eight*, Karrer and Ehrenstein (442). Arginine  $\rightarrow$  ornithine  $\rightarrow$  ornithuric acid  $\rightarrow$   $\alpha$ -benzoylamino- $\delta$ -aminovaleric acid  $\rightarrow$  proline.

It has been reported that proline is racemized by heating it with Ba(OH)<sub>2</sub> but not by refluxing it with 33% sulfuric acid for 24 hours (238). Although treatment with NaOH and acetic anhydride (841) or ketene (414, 582) is ineffective, certain acyl derivatives are rapidly racemized in a mixture of glacial acetic acid and acetic anhydride (141).

*Isolation*. L-Proline is prepared in 15% yield from gelatin by the method of Bergmann (85). Gelatin is hydrolyzed with HCl, arginine is removed as the flavianate, a methanol solution of ammonium rhodanilate is added to the diluted filtrate, the mixture is cooled, the suspension of proline rhodanilate is filtered, the precipitate is washed and recrystallized from methanol containing 0.5 N HCl, the precipitate is dried and mixed thoroughly with aqueous pyridine, the suspension is filtered, and the filtrate is evaporated *in vacuo*. The specific rotation (see Table VI) in water,  $[\alpha]_D^{22} = -85.6^\circ$ , is higher than the value,  $[\alpha]_D^{20} = -80.9^\circ$ , reported by Fischer and Zemplén (314) but is essentially the same as the value,  $[\alpha]_D^{23.4} = -85.0^\circ$ , found by Dunn and Stoddard (unpublished data). Bergmann and Niemann have isolated proline from fibrin (89) and hemoglobin (90) essentially by this procedure. Neuburger (583) has isolated proline as the picrate from the mother liquors obtained in the isolation of hydroxyproline from gelatin but no experimental details were given (see page 312).

In 1901, Fischer (280) isolated proline from casein. An ester fraction (b.p.  $80^\circ$ – $85^\circ$ /8–15 mm.) was boiled in 5 volumes of water for 6–7 hours,

the amino acids were fractionally crystallized from water and from water-ethanol, the filtrate containing most of the proline was evaporated to dryness, and the crude mixture of L- and DL-proline was converted to the copper salts and fractionally crystallized from absolute ethanol. The copper salt of L-proline was found to be more soluble in absolute ethanol than the DL-form. Essentially this method has been employed by Fischer, Abderhalden, Osborne and other workers for the isolation of proline from numerous proteins. In many of these studies the L-proline isolated was impure, or the degree of purity was not established. It has been assumed that the free amino nitrogen detected in purified samples of L-proline (809) resulted from opening of the pyrrolidine ring during the nitrous acid reaction (349) but McCay and Schmidt (539) were able to prepare DL-proline, isolated from the  $\text{Ba}(\text{OH})_2$  hydrolyzate of gelatin and purified by repeated recrystallization as its copper salt, which contained a negligible amount of amino nitrogen.

L-Proline is isolated (as picrate) in about 10% yield from gliadin by the method of Town (795, 797). Gliadin is hydrolyzed with  $\text{H}_2\text{SO}_4$ , the acid is removed as  $\text{BaSO}_4$ ,  $\text{CuCO}_3$  or  $\text{Cu}(\text{OH})_2$  is added to the filtrate, the precipitated copper salts are dried and granulated by treatment with dry acetone, the acetone is removed, and the dry residue is extracted with dry methanol, the methanol is evaporated, the residue is dissolved in water, copper is removed from the solution as  $\text{CuS}$ , the filtrate is evaporated, ethanol is added, the suspension of tyrosine is filtered, an alcoholic solution of cadmium chloride is added, the precipitate is dissolved in water, the cadmium and chloride ions are removed, and picric acid is added to the filtrate. The proline picrate is removed and recrystallized from hot water. The specific rotation in water,  $[\alpha]_D^{18} = -86.7^\circ$  of the L-proline prepared from the picrate, appears to be approximately the correct value at this temperature. According to Cox and King (175), L-proline may be recovered in 90% yield by suspending the picrate in aqueous-aniline solution, extracting the proline solution with ether, decolorizing the aqueous layer with norite, evaporating the filtrate, dissolving the residual material in absolute ethanol and adding ether to the alcoholic solution. The L-proline was found to be free of amino nitrogen, but the rotation was not measured.

Dakin (183) isolated about 9.5% of proline by butanol extraction under reduced pressure of the  $\text{H}_2\text{SO}_4$  hydrolyzate of gelatin. The more insoluble amino acids (leucine, phenylalanine and hydroxyproline) were removed, the butanol was evaporated, the residue was extracted with ethanol, the ethanol was evaporated, the aqueous solution of the residue was treated with mercuric acetate and  $\text{Ba}(\text{OH})_2$  to remove traces of



other amino acids, and the filtrate was evaporated. The specific rotation of the L-proline was  $[\alpha]_D = -79.3$  to  $-82.5^\circ$ . According to the procedure of Kapfhammer and Eck (440), arginine is removed as the flavianate and proline is precipitated first as the reineckate and then as the cadmium chloride complex. The yield from gelatin was 3.7% of L-proline with specific rotation  $[\alpha]_D^{20} = -84.9^\circ$ . L-Proline has been isolated from gelatin as its hydantoin (119) and from ergot (417) and insulin (733) as the gold chloride complex of its betaine.

### 16. Serine

*Synthesis.\** DL-Serine is synthesized in 39% over-all yield by the method of Wood and du Vigneaud (878). Ethyl  $\alpha,\beta$ -dibromopropionate (A) (b.p.,  $98.5-99.5^\circ\text{C./16 mm.}$ ) is prepared in nearly theoretical amount by allowing a mixture of bromine and ethyl acrylate to stand 15 minutes and distilling the product. Ethyl  $\alpha$ -bromo- $\beta$ -ethoxypropionate (B) (b.p.,  $103-104.5^\circ\text{C./13 mm.}$ ) is prepared in 84% yield from equimolar quantities of (A) and sodium ethylate. Since (B) is somewhat unstable and gives off a volatile lachrymator it has been recommended by Dr. du Vigneaud that it be used without purification in the next step. A slight excess of sodium ethylate has also been recommended. DL-Serine is prepared in 47% yield by NaOH hydrolysis<sup>44</sup> of (B), amination of the resulting  $\alpha$ -bromo- $\beta$ -ethoxypropionic acid (C), and HBr hydrolysis of (C). DL-Serine may be synthesized in about the same over-all yield by Carter and West's (142) modification of the method of Schiltz and Carter (681). Methyl  $\alpha$ -bromo- $\beta$ -methoxypropionate (A) is prepared from methyl acrylate, methanol and mercuric acetate through the intermediate  $\alpha$ -acetoxymercuri- and  $\alpha$ -bromomercuri- $\beta$ -methoxypropionates. (A) is hydrolyzed and aminated as described by Wood and du Vigneaud.

DL-Serine is synthesized in 51% over-all yield by Redemann and Icke's (661) modification of the method of Dunn *et al.* (212). Ethoxyacetaldehyde (A) is prepared in aqueous solution by oxidizing ethylene glycol monoethyl ether (ethyl cellosolve) with copper chromite catalyst at  $310-330^\circ\text{C.}$  DL-Serine is prepared by reaction of NaCN,  $\text{NH}_4\text{Cl}$  and  $\text{NH}_3$  in methanol with (A) and hydrolysis of the intermediate ethoxy nitrile with HBr. Other methods for the preparation of ethoxyacetaldehyde have been described by the authors referred to by Dunn (203, p. 21) and by other workers (196-198, 412, 226). This synthesis

\*The synthesis of serine in about 33% over-all yield from methyl acrylate through the intermediates, methyl  $\alpha$ -bromo- $\beta$ -hydroxypropionate and methyl  $\alpha$ -benzylamino- $\beta$ -hydroxypropionate, has been reported by Mattocks and Hartung [*J. Biol. Chem.* 165, 501 (1946)].



of serine is essentially that described by Leuchs and Geiger (501) in 1906.

DL-Serine has been synthesized by the following methods.

*Method One*, Mitra (572). Potassium phthalimide + diethyl bromomalonate  $\rightarrow$  diethyl phthalimidomalonate  $\rightarrow$  diethyl methoxymethyl phthalimidomalonate  $\rightarrow$  serine.

*Method Two*, Mattocks and Hartung (561). Methyl acrylate + HOBr  $\rightarrow$  methyl  $\alpha$ -bromo- $\beta$ -hydroxypropionate  $\rightarrow$  methyl  $\alpha$ -benzoylamino- $\beta$ -hydroxypropionate  $\rightarrow$  serine ester.

*Method Three*, Erlenmeyer (254, 260). Ethyl formate + sodium ethylate  $\rightarrow$  ethyl hippurate  $\rightarrow$  ethyl hydroxymethylene hippurate  $\rightarrow$  benzoylserine ethyl ester  $\rightarrow$  serine.

*Method Four*, Fischer and Leuchs (301). Glycolaldehyde + HCN +  $\text{NH}_3 \rightarrow$  serine.

Daft and Coghill (181) isolated DL-serine by refluxing sericin for 24 hours with 25%  $\text{H}_2\text{SO}_4$ , adding base to pH 9, adding  $\text{H}_2\text{SO}_4$  to neutrality, removing the  $\text{BaSO}_4$  and evaporating the filtrate.

*Isolation*. L-Serine is isolated in 7.5% yield by the method of Stein *et al.* (754, 755). Technically degummed silk is hydrolyzed with HCl, excess HCl is removed as  $\text{PbCl}_2$ , the filtrate is evaporated *in vacuo*, tyrosine is removed and 5-nitronaphthalene-1-sulfonic acid dihydrate is added to the filtrate. The suspension of glycine nitronaphthalenesulfonate is filtered, methyl cellosolve and azobenzene-*p*-sulfonic acid trihydrate are added to the filtrate and the resulting suspension of L-alanine azobenzenesulfonate is filtered. Barium acetate monohydrate is added to the filtrate, the precipitated barium salts are removed, barium ions are removed from the filtrate, the barium-free filtrate is concentrated *in vacuo*, and a mixture of methyl cellosolve and *p*-hydroxyazobenzene-*p'*-sulfonic acid dihydrate is added. The resulting yellow L-serine *p*-hydroxyazobenzene-*p'*-sulfonate is washed and recrystallized from water. The yield of twice recrystallized salt is about 47 g. per 100 g. of technically degummed silk. The L-serine salt is dissolved in water, barium acetate monohydrate is added, the precipitated barium sulfonate is removed, the yellow filtrate is freed of barium and decolorized, the water-clear filtrate is evaporated to dryness *in vacuo*, and the resulting L-serine is recrystallized from water and alcohol. The specific rotations (see Table VI) of the product were  $[\alpha]_D^{28} = -6.8^\circ$  (10% in water),  $[\alpha]_D^{26} = +13.9^\circ$  (10% in *N* HCl),  $[\alpha]_D^{26} = +14.8^\circ$  (10% in 2 *N* HCl) and  $[\alpha]_D^{20} = +15.3^\circ$  (10% in 2 *N* HCl). Fischer and Jacobs found  $[\alpha]_D^{20} = +6.87^\circ$  (1% in water) and  $[\alpha]_D^{28} = -14.3^\circ$  (1% in *N* HCl) for the resolved product.

Serine was isolated from an ester fraction (b.p., 100–125°C./0.5 mm.)

of an HCl hydrolyzate of degummed silk by Fischer and Skita (309) in 1902. Although the product was racemic, essentially this procedure has been used extensively by Fischer, Abderhalden, Osborne, Jones and other workers for the isolation of serine from numerous proteins. Serine has been isolated from proteins as its  $\beta$ -naphthalenesulfonate by Abderhalden (2, 3, 34) and other workers (248, 709, 720).

### 17. Threonine

*Synthesis.* DL-Threonine is synthesized in 4.5% over-all yield by the method of Carter and West (143).  $\alpha$ -Bromo- $\beta$ -methoxy-*n*-butyric acid is prepared in 90% yield by the reaction of methanol, mercuric acetate and crotonic acid (trans). The addition product is dissolved in KBr solution giving a solution of potassium  $\alpha$ -mercuri-bromo- $\beta$ -methoxy-*n*-butyrate (A). Potassium bromide solution and bromine are added to (A), excess bromine is destroyed with sodium bisulfite, and the solution is acidified with HBr and extracted with ether. The crude  $\alpha$ -bromo- $\beta$ -methoxy-*n*-butyric acid is aminated under pressure with concentrated ammonium hydroxide, the solution is concentrated *in vacuo*, acetone is added to the residue, the excess acetone is decanted from the crystalline material and the residue is dissolved in formic acid. Acetic anhydride is added, the solution is evaporated *in vacuo*, the residue is dissolved in water and the solution is cooled overnight. The crystalline mixture of formyl-DL-O-methylthreonine (B) and formyl-DL-O-methylallo-threonine is recrystallized from hot water. The yield of (B), obtained as the precipitate, is 25%. (B) is refluxed with HBr, the solution is evaporated *in vacuo*, the gummy residue is dissolved in absolute ethanol, the solution is neutralized with ammonium hydroxide, the resulting crystals are dissolved in water and 7 volumes of ethanol are added. The yield of DL-threonine, based on (B), is 90%. DL-Allo-threonine is prepared by evaporating the mother liquors from (B) to dryness, refluxing the residue with HBr and recrystallizing the resulting DL-allo-threonine from 50% ethanol. A mixture consisting mainly of allo-threonine was prepared by Carter and Ney (140) from ethyl  $\alpha,\beta$ -dibromobutyrate and sodium methylate through the intermediate,  $\alpha$ -bromo- $\beta$ -methoxybutyrate. The described syntheses of DL-threonine and DL-allo-threonine are based on the earlier studies of Carter *et al.* (137, 854-857, 879), and Abderhalden and Heyns (16). A method for the conversion of DL-allo-threonine to DL-threonine has been described by Carter *et al.* (139). The synthesis of DL-threonine from crotonic acid by the method of Abderhalden and Heyns (16) has been reported (548, 563). Adkins and Reeve (45a) synthesized threonine in 15% over-all yield from ethyl aceto-

acetate through the intermediates ethyl  $\alpha$ -oximinoacetoacetate and ethyl O-ethyl  $\alpha$ -oximinoacetoacetate. Allo-threonine was crystallized from an aqueous solution of the isomers, and threonine was precipitated from the mother liquor by means of ethanol.

*Isolation.* L-Threonine is isolated in 0.08% yield from fibrin by the method of McCoy *et al.* (541). Commercial fibrin is hydrolyzed with  $\text{H}_2\text{SO}_4$ , the acid is removed as  $\text{BaSO}_4$ , the filtrate is evaporated, and the insoluble amino acids are removed, basic cupric carbonate is added, and the precipitated copper salts are removed. Copper is removed from the filtrate as  $\text{CuS}$ , the filtrate is evaporated and extracted repeatedly with butanol, the butanol is removed, the residual amino acids are dissolved in water,  $\text{H}_2\text{SO}_4$  and phosphotungstic acid are added, the precipitate is removed, the filtrate is evaporated, and the precipitate is removed, the  $\text{H}_2\text{SO}_4$  and phosphotungstic acid are removed from the filtrate, ethanol is added and the crude threonine is recrystallized from water and ethanol. The specific rotation of the purified product,  $[\alpha]_D^{25} = -28.2^\circ$ , is in agreement with that,  $[\alpha]_D^{26} = -28.3^\circ$ , found for the resolved product by West and Carter (855).

### 18. Thyroxine

*Synthesis.* DL-Thyroxine is synthesized by the method of Harington and Barger (372) according to the reactions: quinol mono-methyl ether + 3,4,5-triiodonitrobenzene  $\rightarrow$  3,5-diiodo-4-(4'-methoxyphenoxy) nitrobenzene  $\rightarrow$  3,5-diiodo-4-(4'-methoxyphenoxy) aniline  $\rightarrow$  3,5-diiodo-4-(4'-methoxyphenoxy) benzonitrile  $\rightarrow$  3,5-diiodo-4-(4'-methoxyphenoxy) benzaldehyde  $\rightarrow$  azlactone of  $\alpha$ -benzoylamino-3,5-diiodo-4-(4'-methoxyphenoxy) cinnamic acid (A)  $\rightarrow$  ethyl  $\alpha$ -benzoylamino-3,5-diiodo-4-(4'-methoxyphenoxy) cinnamate (B)  $\rightarrow$   $\alpha$ -amino- $\beta$ -[3,5-diiodo-4-(4'-hydroxyphenoxy) phenyl] propionic acid  $\rightarrow$  thyroxine. Harington and McCartney (373) increased the yield of (B) from 25 to 82% by reducing (A) with HI, red P and acetic anhydride. The synthesis of thyroxine reported by Savitzkii (678) is essentially the same as that of Harington and Barger. DL-3,5-Diiodo-4-(3',5'-diiodo-2'-hydroxyphenoxy) phenylalanine, an isomer of thyroxine with one twenty-fifth the activity, has been synthesized by Niemann and Mead (589). Other closely related iodo and fluoro derivatives devoid of physiological activity have been synthesized by Niemann and coworkers (587, 588, 590, 591).

*Isolation.* Thyroxine was first isolated from thyroid glands by Kendall (447, 448) in 1915. Although improvements were made in the isolation procedure in later studies (449-451, 453, 454) the yield of thyroxine was only about 0.5 g. per pound of thyroid. Kendall's thyroxine, as well as

the products isolated by Harington (369) and Foster (320) were optically inactive owing to racemization which occurred during alkaline hydrolysis of the thyroid tissue. L-Thyroxine was isolated from enzymatic hydrolyzates of thyroid by Harington and Salter (379). The specific rotation of the recrystallized material,  $[\alpha]_{5461} = -3.2^\circ$  to  $-4.45^\circ$  (5% solution in mixture containing 2 volumes of ethanol and 1 volume of *N* NaOH) agreed with that,  $[\alpha]_{5461}^{21} = -3.15^\circ$  to  $-4.5^\circ$ , found under the same conditions for the product obtained earlier (370) by resolution of DL-3,5-diiodothyronine through the L-phenylethylamine salt of its formyl derivative and iodination of the resulting L-3,5-diiodothyronine. The specific rotation of the L-thyroxine, isolated by Foster *et al.* (322) essentially by the method of Harington and Salter, was  $[\alpha]_D = -4.4^\circ$ . No highly practicable procedure for the isolation of L-thyroxine has been described.

Physiologically active thyroxine is isolated from the alkaline hydrolyzate of an iodinated protein by the method of Ludwig *et al.* (495, 533, 534). Casein (or other protein) is iodinated, essentially by the method of Hofmeister (404), by the action of iodine at  $40^\circ\text{C}$ . on a mixture of casein and sodium bicarbonate. The product obtained by precipitation with HCl is centrifuged, washed, dissolved in NaOH and dialyzed until free iodine has been removed. The reprecipitated material, containing 7.4% of iodine and 125 guinea pig units per gram, is refluxed with  $\text{Ba}(\text{OH})_2$ , and thyroxine is isolated from the hydrolyzate as its sodium salt. Thyroxine is prepared by dissolving the sodium salt in alkaline 80% ethanol and acidifying the solution with acetic acid. Thyroxine has been prepared by other workers (40, 376, 662) essentially by this procedure. According to Reineke and Turner (663, 664) optically inactive thyroxine, equivalent approximately to 30% of the tyrosine in the protein, is formed by incubating at  $60^\circ\text{C}$ . a mixture of a protein and two atoms of iodine per mole of tyrosine in mildly alkaline solution in the presence of a compound of manganese. The report of von Mutzenbecher (581) that thyroxine is formed by incubating 3,5-diiodotyrosine in alkaline solution has been confirmed by other investigators (69, 102, 371, 377, 378, 429, 664). It has been reported (803, 804) that a synthetic thyroprotein (Protomone) stimulates growth, metabolism, and the production of milk, milk fat and eggs. Although other workers (64, 593) have made similar observations, it has been stated that the practicability of iodinated protein preparations has not been thoroughly established (593).

## 19. Tryptophan

*Synthesis.* DL-Tryptophan is synthesized in 70% over-all yield from crude indole by the method of Albertson and Tullar (50). Ethyl  $\alpha$ -acetamido- $\alpha$ -cyano- $\beta$ -(3-indolyl)-propionate (A) is prepared in 98% yield from ethyl acetamidocyanoacetate<sup>20</sup>, gramine (3-dimethylamino-methyl indole)<sup>43</sup>, sodium, ethanol and ethyl iodide according to the procedure described by Albertson *et al.* (49). DL-Tryptophan is prepared by alkaline hydrolysis of (A). Other practicable syntheses of tryptophan which have been reported recently are outlined in the following equations.

*Method One*, Snyder *et al.* (410, 741). Ethyl acetamidomalonate<sup>17</sup> + gramine methiodide<sup>43</sup> + sodium  $\rightarrow$  ethyl  $\alpha$ -acetamido- $\alpha$ -carbethoxy- $\beta$ -(3-indole)-propionate (A)  $\rightarrow$   $\alpha$ -acetamido- $\alpha$ -carboxy- $\beta$ -(3-indole)-propionic acid  $\rightarrow$  N-acetyltryptophan  $\rightarrow$  tryptophan [81% yield based on (A)].

*Method Two*, Albertson *et al.* (48, 49). Hydrolysis of ethyl  $\alpha$ -acetamido- $\alpha$ -carbethoxy- $\beta$ -(3-indole)-propionate or ethyl- $\alpha$ -carbethoxy- $\alpha$ -benzamido- $\beta$ -(3-indolyl)-propionate essentially by the procedure of Snyder and Smith (74) to give 35% over-all yield of tryptophan based on indole. Smith and Sogn (732) prepared ethyl  $\alpha$ -carbethoxy-3-indole-propionate<sup>45</sup> from ethyl  $\alpha$ -ketocyclopentanone carboxylate (A) but no tryptophan could be synthesized since they were not able to brominate (A). Similarly, Elks *et al.* (245) were unable to brominate ethyl (2-carbethoxy-3-indolylmethyl) malonate in the  $\alpha$ -position although Maurer and Moser (562) stated that they had synthesized tryptophan by this procedure.

Tryptophan was first synthesized in 1907 by Ellinger and Flamand (246, 247) in 17% over-all yield by the method outlined in the following equations. Indole +  $\text{CHCl}_3$  + NaOH  $\rightarrow$  3-indolealdehyde (A). (A) + hippuric acid + sodium acetate + acetic anhydride  $\rightarrow$  azlactone of  $\alpha$ -benzoylamino- $\beta$ -(1-acetyl-3-indole) acrylic acid (B)  $\rightarrow$  3-indole- $\alpha$ -benzoylaminoacrylic acid  $\rightarrow$  tryptophan. Barger and Ewins (66) prepared the homologous  $\alpha$ -amino- $\beta$ -3-methylindole-propionic acid by this method. The synthesis of tryptophan by Bauguess and Berg's (75) modified procedure is indicated by the following equations. (B) + NaOH  $\rightarrow$   $\alpha$ -hydroxy- $\beta$ -3-indoleacrylic acid  $\rightarrow$   $\beta$ -3-indoleacrylic acid  $\rightarrow$   $\alpha$ -oximino- $\beta$ -3-indoleacrylic acid  $\rightarrow$  tryptophan.

The synthesis of tryptophan in 17% over-all yield from 3-indolealdehyde was described by Majima and Kotake (549) in 1922. The method is indicated by the following equations. 3-Indolealdehyde + hydantoin + sodium acetate + acetic anhydride  $\rightarrow$   $\beta$ -indolalhydantoin<sup>46</sup>

<sup>17-20</sup> see p. 360; <sup>43-46</sup> see p. 361.

→  $\omega$ -hydantylskatole → tryptophan. The synthesis of tryptophan by modified hydantoin procedures has been reported by Boyd and Robson (120) and Elks *et al.* (244).

DL-Tryptophan has been prepared by heating the natural form with  $\text{Ba}(\text{OH})_2$  (172, 560), NaOH and acetic anhydride (841), acetic acid and acetyl chloride (852) NaOH and ketene (135, 414), and pyridine (6).

*Isolation.* L-Tryptophan is isolated from casein in 0.7% yield by Cox and King's (176) adaptation of the procedures given by Hopkins and Cole (407), Dakin (182) and Onslow (598). Commercial pancreatin is added to an aqueous suspension of commercial casein, sodium carbonate and sodium fluoride, the suspension is saturated with toluene and the mixture is allowed to stand at 37°C. for 5 days. Additional pancreatin is added, and the mixture is allowed to stand for 12 days. The mixture is shaken daily during the incubation periods. The mixture is placed overnight in the refrigerator and filtered. The precipitate is reserved for the preparation of tyrosine. Sulfuric acid is added to the filtrate, the solution is cooled, a solution of  $\text{HgSO}_4$  in  $\text{H}_2\text{SO}_4$  is added, and the mixture is allowed to stand 48 hours. The supernatant liquid is siphoned, the yellow residual material is washed thoroughly first with a  $\text{H}_2\text{SO}_4$  solution of  $\text{HgSO}_4$  to remove tyrosine and then with distilled water to remove  $\text{H}_2\text{SO}_4$ . The moist precipitate is suspended in water, a hot solution of  $\text{Ba}(\text{OH})_2$  is added until the mixture is alkaline to phenolphthalein, and the mercury is removed as  $\text{HgS}$ . Barium is removed as  $\text{BaSO}_4$ , the filtrate is evaporated *in vacuo*, and the aqueous solution is extracted repeatedly with *n*-butanol. The butanol solution of tryptophan is distilled *in vacuo*, the residual material is cooled and filtered, the crude tryptophan is dissolved in hot 60% ethanol, insoluble material is removed, the ethanol solution is decolorized with norite, and the purified tryptophan is washed with ethanol and ether. The specific rotation in water of analytically pure L-tryptophan is  $[\alpha]_D^{25.0} = -32.15^\circ$  (unpublished data obtained in the writers' laboratory). L-Tryptophan has been isolated from fibrin (91, 336, 586), edestin (28), gliadin (29), yeast (565), lactalbumin (850), silk fibroin (401), salmon muscle (63), and other proteins essentially by this procedure.

## 20. Tyrosine

*Synthesis.* DL-Tyrosine is synthesized in 46% over-all yield by the method of Lamb and Robson (491). 4-(*p*-Methoxybenzylidene)-2-phenyl-5-oxazolone (A) is prepared in 62% yield from anisaldehyde, acetic anhydride, hippuric acid and sodium acetate. The crude product is recrystallized from benzene.  $\alpha$ -Benzoylamino-*p*-methoxycinnamic acid

(B) is prepared in about theoretical amount by refluxing (A) in alcoholic NaOH and neutralizing the solution with  $\text{H}_2\text{SO}_4$ . The crude product is recrystallized from aqueous ethanol. Tyrosine is prepared in 63% yield by refluxing (A) with a mixture of glacial acetic acid, constant boiling HI and red phosphorus. The reaction mixture is filtered, the precipitate is washed with hot acetic acid, the combined filtrate and washings are evaporated to dryness *in vacuo*, the residue is dissolved in water, the solution is decolorized with norite, and the filtrate is concentrated by evaporating it *in vacuo*. The reduction and hydrolysis of (A) with HI was carried out essentially in the manner recommended by Harington and McCartney (373). The synthesis of tyrosine from *p*-hydroxybenzaldehyde through the intermediate azlactone has been described by Erlenmeyer and Halsey (257, 258), Fischer (277) and Latham (494).

Tyrosine may be synthesized satisfactorily by the following methods.

*Method One*, Hamlin and Hartung (366). Ethyl *p*-methoxyphenyl acetoacetate + butyl nitrite  $\rightarrow$  ethyl *p*-methoxyphenyl- $\alpha$ -oximinopropionate  $\rightarrow$  O-methyltyrosine  $\rightarrow$  tyrosine. The over-all yield is 64%.

*Method Two*, Sasaki (677). Glycine anhydride<sup>47</sup> + anisaldehyde + sodium acetate + acetic anhydride  $\rightarrow$  3,5-dianisal-2,5-diketopiperazine  $\rightarrow$  3,5-dianisyl-2,5-diketopiperazine  $\rightarrow$  tyrosine. The over-all yield is 48%.

*Method Three*, Stephen and Weizmann (756). Diethyl potassium phthalimidomalonate + anisyl bromide  $\rightarrow$  diethyl *p*-methoxybenzylphthalimidomalonate  $\rightarrow$  tyrosine.

*Method Four*, Wheeler and Hoffman (859) and Johnson *et al.* (424, 426). Hydantoin + sodium acetate + acetic acid + anisaldehyde  $\rightarrow$  anisalhydantoin  $\rightarrow$  anisylhydantoin  $\rightarrow$  tyrosine. The over-all yield is 65%.

Other syntheses of tyrosine have been reported by methods indicated by the following equations.

*Method Five*, Feofilaktov *et al.* (275). *p*-Methoxybenzene + formaldehyde +  $\text{ZnCl}_2$  + HCl  $\rightarrow$  *p*-methoxybenzyl chloride (A). (A) + ethyl acetoacetate + sodium  $\rightarrow$  ethyl *p*-methoxybenzylacetoacetate (B). (B) + phenyl diazonium chloride  $\rightarrow$  *p*-methoxyphenylpyruvic acid phenylhydrazone  $\rightarrow$   $\alpha$ -amino- $\beta$ -*p*-methoxyphenylpropionic acid  $\rightarrow$  tyrosine. An over-all yield of 27% was reported.

*Method Six*, Gaudry (340). Acrylonitrile + *p*-methoxyphenyldiazonium chloride  $\rightarrow$   $\alpha$ -chloro- $\beta$ -*p*-methoxyphenylpropionitrile  $\rightarrow$   $\alpha$ -chloro- $\beta$ -*p*-methoxyphenylpropionic acid  $\rightarrow$  tyrosine. The over-all yield was 6%.

DL-Tyrosine is prepared by heating the active form with  $\text{Ba}(\text{OH})_2$  (238), NaOH (848, 869), NaOH and acetic anhydride (840), acetic acid

<sup>47</sup> see p. 361.



and acetyl chloride (852), and acetic acid and acetic anhydride (96).

*Isolation.* L-Tyrosine is isolated from casein in 3% yield by the method of Cox and King (176). The precipitate reserved for the preparation of tyrosine (tryptophan procedure, page 331) is suspended in 2.5 N HCl, the suspension is boiled for 30 minutes and strained, the filtrate is heated with norite, the hot suspension is filtered, and the warm filtrate is extracted with benzene to remove fat which retards filtration. The aqueous solution is heated to boiling, the solution is neutralized with ammonia, the mixture is allowed to stand overnight in the refrigerator and the suspension is filtered. The crude tyrosine is dissolved in NaOH solution, the solution is decolorized with norite, the suspension is filtered and the hot solution is neutralized with HCl. The mixture is allowed to stand overnight in the refrigerator, the suspension is filtered and the crystals are washed until the washings are free of chloride. The specific rotation of pure tyrosine in 6.3 N HCl is  $[\alpha]_D^{20} = -8.64^\circ$  (277) and in 6.08 N HCl is  $[\alpha]_D^{25.6} = -7.27^\circ$  (see Table VI).

Cox *et al.* (177) isolated 22 g. of L-tyrosine from 4 kg. of blood corpuscle paste (containing 5.80% nitrogen and 36.2% solids). The paste was hydrolyzed with sulfuric acid, the acid was removed as BaSO<sub>4</sub>, the filtrate and washings were concentrated *in vacuo*, the residual liquid was allowed to stand for 2 days in the refrigerator, and the suspension was filtered. The crude precipitate was boiled with glacial acetic acid to dissolve leucine, the residual crude tyrosine was extracted with boiling glacial acetic acid, the purified tyrosine was dissolved in NaOH solution, the solution was decolorized with norite, and tyrosine was precipitated from the filtrate with HCl. The resulting tyrosine was washed until free of HCl.

Tyrosine was first isolated in 1846 by Liebig (516, 517) from the KOH fusion products of casein. The isolation of tyrosine from HCl and H<sub>2</sub>SO<sub>4</sub> hydrolyzates of casein and other proteins was first reported by Bopp (115) in 1849. Since that date tyrosine has been isolated by a variety of procedures from casein (33, 70, 119, 166, 167, 189, 280, 292, 319, 332, 399, 551, 611, 638, 792), silk (4, 5, 9, 32, 92, 119, 290, 308, 423, 763, 802, 885), hair (38, 132, 575, 884), fibrin (35, 91, 164, 165, 250, 251), keratins (21, 22, 130, 131, 858), egg proteins (24, 506, 730), lactalbumin (25, 434), potato proteins (726), wheat proteins (29, 602), corn proteins (127, 184), hemp seed (3, 12), soy proteins (606, 683), pea proteins (512, 608, 613, 615), flax (512), squash (607, 699), lupine sprouts (695, 697), pumpkin seed (8), kidney bean (603), almond (609), barley (605), cottonseed (27, 28), sunflower seed (26), wool (34), Brazil nut (604), coconut (433), peanut (419), castor bean (443), yeast (565), beef spinal cords (37), salmon muscle (63), and numerous other proteins.



## 21. Valine

*Synthesis.* DL-Valine is synthesized in 42% over-all yield by the method of Marvel (553). DL- $\alpha$ -Bromoisovaleric acid (A) is prepared in 88% yield by refluxing a mixture of isovaleric acid, bromine and phosphorus trichloride. The crude product (b.p., 110–125°C./15 mm.) is distilled *in vacuo*. A mixture of (A) and concentrated ammonium hydroxide is allowed to stand at room temperature for a week. Excess ammonia is removed by distilling the mixture, and the resulting solution is distilled *in vacuo* to a thin paste. The residual material is filtered, the precipitate is dissolved in water, the solution is decolorized with norite, the suspension is filtered, and an equal volume of 95% ethanol is added to the hot filtrate. The crude valine is washed with absolute ethanol. A second crop is obtained by working up the filtrates. This procedure is essentially the same as that described by Clark and Fittig (157), Schlebusch (682), Schmidt and Sachtleben (686), and Slimmer (731).

DL-Valine may be synthesized in 40% over-all yield essentially by the method of Lipp (520). The cost of valine prepared by this procedure is relatively low. Freshly distilled isobutyraldehyde (1 mole) is added dropwise with stirring to a mixture of ice-cold ammonium hydroxide (1.5 moles) and chopped ice. The mixture is allowed to warm to room temperature, ice-cold anhydrous liquid HCN<sup>2</sup> (1.5 moles) is added slowly with stirring and concentrated HCl is added to the reaction mixture. HCN is removed by heating the solution, and the liquid is evaporated to dryness under reduced pressure. The residue is dissolved in hot 95% ethanol, the solution is cooled, the suspension of NH<sub>4</sub>Cl is filtered, and the precipitate is washed thoroughly with 95% ethanol. The combined filtrate and washings are brought to pH 6 with concentrated ammonium hydroxide, the solution is evaporated to dryness under reduced pressure, the residue is dissolved in hot glacial acetic acid, the mixture is cooled, the suspension of NH<sub>4</sub>Cl is filtered, the precipitate is washed thoroughly with cold glacial acetic acid, the combined filtrate and washings are evaporated to dryness *in vacuo*, the residue is triturated with hot absolute methanol, the suspension is filtered, and the precipitate is dried.

DL-Valine is synthesized in 43% over-all yield by the method of Albertson and Tullar (50). Ethyl  $\alpha$ -acetamido- $\alpha$ -cyano- $\beta$ -methylbutyrate (A) is prepared in 66% yield from ethyl acetamidocyanoacetate<sup>20</sup> and isopropyl bromide. DL-Valine is prepared in 65% yield by the alkaline hydrolysis of (A).

Other methods by which DL-valine has been synthesized are indicated by the following equations.

*Method One*, Curtius and Benckiser (180). Diethyl isopropylmalonate

→ potassium ethyl isopropylmalonate → potassium isopropylmalonylhydrazide → isopropylmalonylazide → polymeric valine anhydride → valine.

*Method Two*, Redemann and Dunn (660). Diethyl benzamidomalonate + sodium + ethanol + isopropyl iodide → diethyl  $\alpha$ -benzamido- $\alpha$ -isopropylmalonate → valine. The over-all yield is 47%.

*Method Three*, Feofilaktov (266) and Feofilaktov and Zaitsev (273, 274). Potassium phenyldiazotate + isopropylacetoacetic ester → isobutylformic acid phenylhydrazone → valine. The over-all yield was 53%.

DL-Valine has been prepared by heating a mixture of  $\text{Ba}(\text{OH})_2$  and the active form of valine for 2 days at 160–170°C. (280)..

*Isolation*. L-Valine was first isolated from lupine sprouts by Schulze and Barbieri (701) in 1883. Asparagine, proteins and leucine were removed, and valine was separated from phenylalanine by fractional crystallization of their copper salts. This procedure was used by Schulze (694, 695, 697) in the isolation of valine from various types of plant sprouts. Essentially this method has been used by Fischer and Dorpinghaus (296), Abderhalden, Osborne, Jones, Levene and Van Slyke, Brazier, Dakin, and other workers who have isolated valine from a variety of proteins. The amino acids were separated by fractionally crystallizing glutamic acid as its hydrochloride, tyrosine and leucine as the free amino acids, diamino acids as their phosphotungstates, and glycine as its ester hydrochloride and by fractionally distilling the esters of the monoamino acids. Valine was separated from leucine, phenylalanine and other amino acids by fractionally crystallizing the copper, lead and zinc salts of these amino acids. The optical purity of most of the products isolated either was not determined or was low as judged by the specific rotation in 6.0 N HCl,  $[\alpha]_D^{20} = +28.8^\circ$ , found by Fischer (286) for the resolved product. A thoroughly satisfactory method for the isolation of valine is not available at the present time.

### III. RESOLUTION

The optical isomers of DL-histidine have been separated by the fractional crystallization (spontaneous resolution) procedure described by Duschinsky (227) but no other applications of this method have been reported. DL-Amino acids are commonly resolved by fractional crystallization from water, ethanol or methanol of the salts formed from the DL-amino acid or a derivative and an optically active form of an organic acid or base. The more insoluble salt is removed and hydrolyzed, and the optically active amino acid (A) is purified by recrystallizing it from

water, water-ethanol or other solvent. The more-soluble salt present in the mother liquor is hydrolyzed, and the isomeric amino acid (B) is recovered. This antipode (B) is usually purified by fractional crystallization of its salt formed from an optically active organic acid or base of different type but of the same configuration as that employed for the isolation of (A). Purification of (B) may also be effected by crystallization of its salt formed from an optically active organic acid or base of the same type but of the opposite configuration as that used to isolate (A). The general principles governing the resolution of racemic mixtures have been discussed by Shriner *et al.* (721).

Both optical isomers of a DL-amino acid are oxidized to  $\alpha$ -keto acids and other products by the action of molds, yeasts, bacteria and amino acid oxidases but one form of some of the amino acids is oxidized more rapidly than the other. Either the D- or L-amino acid may be isolated from the reaction products depending upon the type of organism or amino acid oxidase employed. Procedures for the preparation of various D- and L-amino acids in this manner are outlined in the footnotes to Table I. The literature on this topic prior to 1922 has been reviewed by Ehrlich (239).

A summary of methods by which amino acids have been resolved is given in Table I.

TABLE I  
*Resolution of Amino Acids\**

Amino Acid	Derivative	L-amino Acid	Alkaloid or Derivative for D-amino Acid	References
Alanine <sup>a</sup>	benzoyl	brucine	strychnine	276
	benzoyl	strychnine	brucine	225, 285, 513, 623, 641, 727
	<i>m</i> -nitrobenzoyl	quinine	brucine	169
	benzenesulfonyl	ephedrine	....	341
	ethyl ester	....	hydroxymethylenecamphor	460
	$\alpha$ -naphthalenesulfonyl	brucine	strychnine	168
	$\beta$ -naphthalenesulfonyl	strychnine	....	168
	ethyl ester	....	bromocamphor sulfonic acid	170
	<i>p</i> -toluenesulfonyl	strychnine	brucine	342
	menthoxyacetyl	....	....	406
Aspartic acid <sup>b</sup>	benzoyl	brucine	brucine	276
Cystine	....	....	....	800
	acetyl	brucine	cholestanonesulfonic acid	405, 833, 834
	formyl	strychnine	strychnine	834, 838
	formyl	brucine	brucine	528
Glutamic acid <sup>c</sup>	N-formyl-S-benzyl-DL-cysteine	....	brucine	876
	benzoyl	strychnine	strychnine	276
	DL-pyroglutamic acid	quinine	quinine	767
	....	tartaric acid	tartaric acid	649
Histidine <sup>d</sup>	phenylisocyanate	quinine	quinine	498
Hydroxyproline	ethyl ester	brucine	brucine	523
Isoleucine <sup>e</sup>	formyl	brucine	brucine	39
	menthoxyacetyl	....	....	485

<sup>a-e</sup> see p. 339

TABLE I  
Resolution of Amino Acids\* (Continued)

Amino Acid	Derivative	L-amino Acid	Alkaloid or Derivative for D-amino Acid	References
Leucine <sup>f</sup>	benzoyl formyl ....	quinidine brucine ....	cinchonine brucine cholestenonesulfonic acid	278 310 800
Lysine	....	D-camphoric acid	L-camphoric acid	83, 84, 793
Methionine <sup>g</sup>	formyl	brucine	brucine	413, 867
Phenylalanine <sup>h</sup>	formyl	cinchonine	cinchonine	307, 837, 840
Proline <sup>i</sup>	benzoyl	cinchonine	cinchonine	302
Serine <sup>j</sup>	m-nitrobenzoyl p-nitrobenzoyl phosphoric acid	brucine	quinine	314
Threonine	formyl-O-methyl	....	brucine	181, 299
Tryptophan	acetyl	brucine	brucine	510
	acetyl	α-phenylethylamine	α-phenylethylamine	855
Tyrosine <sup>k</sup>	benzoyl	quinine	quinine	841, 842
	formyl	brucine	cinchonine	82
	....	....	brucine	277
Valine <sup>m</sup>	formyl	brucine	cholestenonesulfonic acid	30
	....	menthoxyacetyl	brucine	800
			menthoxyacetyl	286
				406

<sup>f-m</sup> see p. 339.

## FOOTNOTES TO TABLE I

\* D-Arginine nitrate has been prepared by Riesser (666) by incubating a mixture containing DL-arginine carbonate and fresh liver extract for 20 hours at 35°C.

<sup>a</sup> The optical isomers of alanine have been prepared by amination of D- and L- $\alpha$ -bromopropionic acids. The latter have been prepared by the action of  $\text{PCl}_5$  and acetyl chloride on L-serine methyl ester hydrochloride (304), by the action of nitrosyl bromide on L-alanine (289), and by the resolution of DL- $\alpha$ -bromopropionic acid (147, 654-656, 507, 845). The preparation of D-alanine by the action of yeast on DL-alanine has been described by Ehrlich (234). L-Alanine has been prepared by the action of takadiastase (408, 584) and D-amino acid oxidase (79, 229) obtained from sheep or hog kidneys. The last author (Behrens) isolated 22 g. of nearly pure L-alanine from the reaction products of 72 g. of DL-alanine. The velocity of the oxidation of D-alanine by D-amino acid oxidase has been determined by Stadie and Zapp (752).

<sup>b</sup> L-Aspartic acid has been prepared by the reaction of fumaric acid and ammonia in the presence of *E. coli* (651).

<sup>c</sup> D-Glutamic acid has been prepared by the action of molds (568, 645, 702) and yeast (238) on DL-glutamic acid and of papain-cysteine on carbobenzoxy-DL-glutamic acid anilide (88, 334).

<sup>d</sup> D-Histidine has been prepared by the action of yeast (36, 238) on DL-histidine, by spontaneous resolution of DL-histidine (227) and by isolation from the urine of animals fed DL-histidine (36, 464).

<sup>e</sup> D-Isoleucine has been prepared by the action of yeast on DL-isoleucine (238).

<sup>f</sup> D- and L-leucine have been prepared by amination of D- and L- $\alpha$ -bromoisocaproic acid (295) and by the action of pancreatin on DL-leucine (846). L-Leucine has been prepared by the action of *P. glaucum* on DL-leucine (702, 704). D-Leucine has been prepared by the action of L-amino acid oxidase on DL-leucine (766).

<sup>g</sup> D-Methionine has been prepared by the action of L-amino acid oxidase on DL-methionine (766). L-Methionine has been prepared by the action of D-amino acid oxidase on DL-methionine (229).

<sup>h</sup> D-Phenylalanine has been prepared by the action of yeast (236) and L-amino oxidase (766) on DL-phenylalanine. D- and L-Phenylalanines have been prepared by the action of ammonia on L- and D- $\alpha$ -bromohydrocinnamic acids, respectively (295), and by the action of papain-cysteine on acetyl-DL-phenylalanylglycine anilide (80, 88).

<sup>i</sup> L-Proline has been prepared by the action of D-amino acid oxidase on DL-proline (753).

<sup>j</sup> D-Serine has been prepared by the action of yeast on DL-serine (236).

<sup>k</sup> D-Tyrosine has been prepared by the action of pancreas extract on DL-tyrosine ethyl ester (31) and by the action of yeast (240, 241) and *Oidium lactis* (152) on DL-tyrosine.

<sup>m</sup> D-Valine has been prepared by the action of yeast on DL-valine (242).

## IV. SYNTHESIS OF AMINO ACIDS CONTAINING ISOTOPIC ATOMS

Amino acids containing the isotopic atoms D,  $\text{N}^{15}$ ,  $\text{S}^{34}$ ,  $\text{S}^{35}$  and  $\text{C}^{13}$  have been synthesized from  $\text{D}_2$ ,  $\text{D}_2\text{O}$ ,  $\text{D}_2\text{SO}_4$ ,  $\text{CD}_3\text{OH}$ ,  $\text{N}^{15}\text{H}_3$ ,  $\text{H}_2\text{S}^{34}$ ,  $\text{H}_2\text{S}^{35}$ ,  $\text{C}^{13}\text{H}_4$ ,  $\text{NaC}^{13}\text{N}$ , and other compounds. Compounds with stable D and

$N^{15}$  constituents are available from commercial sources while those with the relatively unstable isotopic sulfur and carbon atoms have been prepared by bombardment of  $CCl_4$  [to give  $S^{35}$  (777)] and other compounds with neutrons (439).

Isotopic atoms have been introduced into amino acids or molecules employed in the synthesis of amino acids, by (a) catalytic addition of D to carbon-carbon double bonds (161, 459, 758, 852), (b) methylation with trideuteromethyl iodide (831, 833), (c) syntheses of deuteromalonic ester derivatives from  $\omega$ -phenyl deuterioalkyl bromides and malonic ester (626), (d) preparation of deuterio amino acids by reaction of amino acids with deuteriosulfuric acid (576, 667, 692, 758, 805, 852), (e) catalytic reduction with  $D_2O$  and amination of  $\alpha$ -keto acids (668), (f) preparation of deuterioaliphatic acids by reaction of aliphatic acids with  $D_2O$  (690), (g) catalytic reduction and amination with  $N^{15}H_3$  of  $\alpha$ -keto acids (690, 832), (h) reaction of phthalic acid with  $N^{15}H_3$  to give  $N^{15}$ -phthalimide (690, 757), (i) bombardment of  $CCl_4$  with neutrons to give  $S^{35}$ , conversion of  $S^{35}$  to  $H_2S^{35}$  and  $S^{35}$ -benzylmercaptan (A) and synthesis of amino acids through (A) (714, 777, 779), (j) reaction of Grignard reagents with  $C^{13}O_2$  to give aliphatic acids containing  $C^{13}$  in the  $COOH$  group (597), and (k) reaction of  $NaC^{13}N$  to give  $CH_3C^{13}N \rightarrow CH_3C^{13}H_2NH_2 \rightarrow CH_3C^{13}H_2N^+(CH_3)_3OH^- \rightarrow C^{13}H_2=CH_2 \rightarrow C^{13}H_2Cl-CH_2Cl$ . Reaction of (A) with  $C_6H_5CH_2S^{34}H$  to give  $C_6H_5CH_2S^{34}-H_2CH_2Cl$  (B) and synthesis of amino acids by reaction of (B) and sodium phthalimidomalonate (457).

The isotopic amino acids which have been synthesized are deuterio-DL-alanine (667, 692),  $N^{15}$ -DL-alanine (690),  $N^{15}$ -DL-aspartic acid (690),  $S^{35}$ -DL-cystine (714),  $C^{13}$ - and  $S^{34}$ -DL-benzylhomocysteine [ $C_6H_5CH_2-S^{34}C^{13}H_2-CH_2CH(NH_2)COOH$ ] (457), tetradeutero-DL-homocysteine (626),  $S^{35}$ -DL-homocysteine (885), dideutero-DL-glutamic acid (668),  $N^{15}$ -DL-glutamic acid (690),  $C^{13}$ -glycine [ $NH_2CH_2C^{13}OOH$ ] (597),  $N^{15}$ -glycine (690), trideutero-DL-methionine [ $CD_3SCH_2CH_2CH(NH_2)COOH$ ] (831, 833),  $\beta,\gamma$ -dideutero-DL-methionine (626),  $S^{35}$ -DL-methionine (714, 777, 779),  $C^{13}$ - and  $S^{34}$ -DL-methionine [ $CH_3S^{34}C^{13}H_2CH_2CH(NH_2)COOH$ ] (457), deuterio-DL-leucine (459, 667, 692, 805) deuterio-L-leucine (805),  $N^{15}$ -deutero-DL-leucine (690),  $N^{15}$ -deutero-D-leucine (659, 691),  $N^{15}$ -deutero-L-leucine (691),  $N^{15}$ - $\beta,\gamma,\delta$ -trideutero-DL-lysine (852),  $N^{15}$ - $\beta,\gamma,\delta$ -trideutero-L-lysine (852),  $N^{15}$ -DL-norleucine (690), deuterio-DL-ornithine (161), deuterio-DL-phenylalanine (576),  $N^{15}$ -DL-phenylalanine (690),  $N^{15}$ -DL- $\alpha$ -amino- $\gamma$ -phenylbutyric acid (832),  $N^{15}$ -L- $\alpha$ -amino- $\gamma$ -phenylbutyric acid (832),  $N^{15}$ -D- $\alpha$ -amino- $\gamma$ -phenylbutyric acid (832),  $N^{15}$ - $\beta,\gamma,\delta$ -trideutero-DL-proline (758),  $N^{15}$ - $\beta,\gamma,\delta$ -trideutero-L-

proline (758), N<sup>15</sup>-DL-serine (757), N<sup>15</sup>-DL-tyrosine (690), and  $\beta,\gamma$ -dideutero-DL-valine (459).

Metabolism studies using D- and N<sup>15</sup> amino acids as tracer substances have been reviewed by Schoenheimer (689). The use of radiosulfur in animal metabolism has been discussed by Tarver and Schmidt (778). Isotopic amino acids with deuterium substituted in the amino or carboxyl group or on a carbon atom from which rearrangement of deuterium to oxygen or other similar atoms might take place are not suitable for use in tracer studies. The exchange of deuterium in deuterioamino acids has been investigated by Rittenberg *et al.* (667). Methods have been described for the determination by the isotope dilution procedure of a number of amino acids in serum albumin (719),  $\beta$ -lactoglobulin (321) and hemoglobin (321). The general applicability of the isotope dilution method has been discussed by Henriques and Margnetti (391).

#### V. PURIFICATION

The analytically pure amino acids required for the measurement of physical properties of amino acids, the determination of amino acids by colorimetric and microbiological methods and the study of the metabolic functions of amino acids, may be prepared by recrystallization from water, dilute HCl, or an aqueous solution of methanol, ethanol or other alcohol. An amino acid is recrystallized under conditions such as those outlined in Table II until inorganic impurities have been removed, and the value found for a physical property (such as specific rotation or solubility) or a constituent (such as nitrogen) is in close agreement with the standard or theoretical figure. If no standard figure for a physical property is available the product is recrystallized until a constant value is obtained.

*Note added by Editors.* Among the impurities difficult to remove from amino acid preparations are generally inorganic salts; for both the salts and the amino acids are far more soluble in water than in organic solvents. The salts are present in solution, however, as ions; the amino acids—at least those of the monamino monocarboxylic type, in the pH range 5 to 8—almost entirely as dipolar ions of zero net charge. Taking advantage of these facts, N. R. Joseph (*J. Biol. Chem.* 126, 403, 1938) has purified amino acids by electrodialysis, using a mercury anode and a mercury cathode, separated from the amino acid solution by cellophane membranes. Dilute HCl solutions are placed above the mercury, in both anode and cathode chambers, with platinum electrodes dipping into the acid. Joseph was able to remove ionic impurities from glycine and  $\beta$ -alanine solutions, to an extent equivalent to several recrystallizations, after passage of a current for several hours. The loss of amino acid from the solution was very small. It appears possible that other ionophoretic techniques, discussed in detail by Martin and Synge in Vol. II of this publication as tools of amino acid analysis, may also be used effectively (with suitable modifications) for amino acid purification.



Special care must be taken in the purification of amino acids if the final products are to be analytically pure. Some of the precautions and special techniques considered to be important are mentioned in the following discussion. The recommended solvents include C.P. HCl and  $\text{NH}_4\text{OH}$ , distilled water which has been redistilled from potassium permanganate in an all-glass apparatus and redistilled best-grade methanol and ethanol. Decolorizing carbon (norite or Nuchar) should be boiled with hot 6 *N* HCl to remove impurities and finally washed with boiling distilled water to remove HCl. Only sintered-glass funnels or the Hirsch- or Buchner-type funnels equipped with special (low ash, high wet strength, acid washed, lintless) filter paper such as Eaton-Dikeman's Lintless Filter Papers, No. 852-4 should be used in filtering amino acid solutions and suspensions.

Every effort should be made to avoid contamination of the amino acid with foreign materials. Amino acids should be recrystallized only from scrupulously cleaned flasks which are covered so far as possible at all times. Solutions and suspensions are mixed by shaking or rotating the flask rather than by stirring. Suspensions of carbon in solutions which have been decolorized are filtered until no trace of carbon is visible on the filter paper or sintered-glass funnel. Hot, nearly saturated solutions of amino acids are filtered conveniently with steam-heated, jacketted funnels of the Coors Company's porcelain Buchner type or the Corning Glass Company's sintered-glass type.

In crystallizing amino acids, the hot solution is allowed to cool in air to room temperature while shaking the flask at intervals to prevent caking of the crystals and to permit formation of crystals having uniform, relatively large size. The suspension is placed overnight in the refrigerator and filtered, the precipitate is washed thoroughly, and the product is recrystallized until it is analytically pure. A minimum of three and a maximum of ten recrystallizations may be required. Washing of precipitates is usually continued until the test is negative for a known impurity (such as ammonia or chloride ion) in a 1-ml. aliquot of the final wash water.

The final precipitate, sucked as dry as possible, is blown into a clean glazed porcelain evaporating dish by a gentle current of air applied through the neck of the funnel. Any material which clings to the funnel is discarded or preserved with other residues for recrystallization. The evaporating dish, covered with a clean watch glass, is placed overnight in an oven at 45–50°C., and the oven-dried product is allowed to stand 24 hours in air to bring it into equilibrium with atmospheric moisture. This material is transferred to a number of scrupulously cleaned, dry

bottles which are kept tightly stoppered in order to minimize chances of contamination.

A summary of the conditions employed in the writers' laboratory for the purification of the amino acids listed is given in Table II.

TABLE II

*Summary of Conditions Employed for Purification of Amino Acids*

Amino Acid	Solvent 1		Solvent 2		Wash Fluid(d)
	Name	ml./g. Amino Acid	Name	ml./g. Amino Acid	
DL-Alanine	Water	3.0	95% Ethanol	6.0	95% Ethanol
L-Alanine	Water	3.0	95% Ethanol	6.0	95% Ethanol
L-Arginine	Water	1.2	....	...	40-95% Ethanol
L-Arginine · HCl	0.4 N HCl(a)	0.5	....	...	80-95% Ethanol
DL-Aspartic acid	Water	9.5	....	...	Ice water
L-Aspartic acid	Water	25.0	....	...	Ice water
L-Cystine	1.5 N HCl	18.0	0.6 N NH <sub>4</sub> OH to pH 5	...	Hot water
DL-Glutamic acid · H <sub>2</sub> O	Water(b)	9.0	....	...	20-50% Ethanol
L-Glutamic acid	Water	12.0	....	...	50-100% Methanol
Glycine	Water	2.3	95% Ethanol	1.0	35-95% Ethanol
L-Histidine	Water	6.0	....	...	50-95% Ethanol
L-Histidine · HCl · H <sub>2</sub> O	0.2 N HCl(a)	1.0	....	...	75-95% Ethanol
L-Hydroxyproline	{ 50% Methanol } { 50% Ethanol }	3.1	....	...	Methanol
DL-Isoleucine	Water	17.5	95% Ethanol	4.4	25-95% Ethanol
DL-Leucine	Water	30.0	....	...	25-100% Methanol
L-Leucine(e)	Water	24.0	95% Ethanol	24.0	50-95% Ethanol
DL-Lysine · HCl	0.2 N HCl(a)	1.0	....	...	60-95% Ethanol
L-Lysine · HCl	0.2 N HCl(a)	1.0	....	...	60-95% Ethanol
DL-Methionine	Water	9.0	95% Ethanol	9.0	50-95% Ethanol
L-Methionine	Water	17.0	95% Ethanol	34.0	80% Ethanol
DL-Phenylalanine	Water	23.0	95% Ethanol	15.0	95% Ethanol
L-Proline	Abs. ethanol(c)	15.0	....	...	Abs. ethanol
DL-Serine	Water	13.0	95% Ethanol	4.5	30-95% Ethanol
DL-Threonine	Water	2.5	95% Ethanol	20.0	95% Ethanol
L-Tryptophan	65% Ethanol	42.0	....	...	95% Ethanol
DL-Tyrosine	Water	220.0	....	...	Ice water
L-Tyrosine	Water	200.0	....	...	Ice water
DL-Valine	Water	7.0	95% Ethanol	7.0	50-95% Ethanol

<sup>a</sup>HCl is used to depress hydrolysis of the amino acid hydrochloride. L-Lysine HCl · 2H<sub>2</sub>O crystallizes from the HCl solution but the water of crystallization is lost at 45°C.

<sup>b</sup>A maximum temperature of 80°C. is employed to minimize the conversion to pyroglutamic acid. The aqueous solution is seeded with DL-Glutamic acid · H<sub>2</sub>O immediately before adding ethanol to insure crystallization of the stable monohydrate; otherwise, a mixture of the anhydrous and monohydrated form crystallizes.

<sup>c</sup>A mixture containing 75% *n*-propanol and 25% ethanol (95%) may be used.

<sup>d</sup>The first washing is made with the solvent of lowest concentration and the last with that of the highest concentration.

<sup>e</sup>Only products purified by one of the methods described on page 315 should be used. Initial purification by recrystallizing the formyl derivative is less satisfactory than the other methods since the procedure is tedious, and the yield is low.

## VI. CRITERIA OF PURITY

Adequate evidence pointing to the high degree of purity of a given sample of any amino acid is required before it can be considered to be nearly analytically pure even though the material may have been subjected to numerous recrystallizations. It seems necessary in most cases, therefore, to establish within acceptable limits: 1) freedom from ammonia, the expected types of inorganic ions and non-volatile ash; and 2) the moisture, total or amino nitrogen, and carboxyl content as minimum criteria of purity. In special cases, the determination of equivalent weight by glass electrode titration with standard base, specific rotation and solubility and microbiological tests are considered to be desirable and may be obligatory. It is evident that the degree of purity found for a given sample of an amino acid may be considered established only within the limits of accuracy of the procedures and techniques employed.

An amino acid sample thought to be pure should be a pure-white solid devoid of any suggestion of color and it should yield a crystal-clear aqueous or HCl solution free entirely from specks of carbon, shreds of filter paper or toweling, or other visible insoluble impurities. It has been found futile to analyze further any sample which does not conform to this standard. Subjection of the sample to the qualitative tests described in a later section has been found to be an informative second step in the analysis. If the proportions of ammonia and the inorganic ions are below the allowable limits it can be reasonably expected that the high purity of the product will be borne out by the subsequent analyses. The presence of an excessive quantity of any one of these impurities indicates that the product should be further purified.

Descriptions and limitations of various analytical procedures are given below.

### 1. *Semi-quantitative Tests for Ammonia, Iron, Chloride, Phosphate and Heavy Metals*

The procedures, described by Stoddard and Dunn (759) are convenient, rapid and quite reliable for all amino acids examined except the special cases noted. These tests, employed routinely by Amino Acid Manufacturers since 1935, have been found to be useful preliminary criteria of purity of amino acids. The qualitative tests found to be satisfactory are indicated by + signs in Table III. Unsatisfactory tests are indicated by — signs. Tests not performed are indicated by .. signs.

### 2. *Quantitative Analysis*

*a. Moisture.* In the determination of moisture, a glazed porcelain crucible is heated for 5 hours at 70°C. and 10 mm. in a vacuum oven in the presence of fresh anhydrous calcium chloride. The crucible is

TABLE III  
*Summary of Qualitative Tests on Amino Acids*

Amino Acid	Ammonia	Iron	Chloride	Phosphate	Heavy Metals
Alanine	+	+	+	+	+
Arginine	..	+	+	+	+
Arginine • HCl	A	+	..	+	+
Aspartic acid	+	+	+	+	+
Cystine	—	+	+	E	—
Cysteine	+	+	+	F	+
Cysteine • HCl	+	+	..	F	+
3,5-Diiodotyrosine	+	—	—	—	—
Glutamic acid	+	+	+	+	+
Glycine	+	+	+	+	+
Histidine	B	+	+	+	+
Histidine • HCl • H <sub>2</sub> O	B	+	..	+	+
Hydroxyproline	+	+	+	+	+
Isoleucine	+	+	+	+	+
Leucine	+	+	+	+	+
Lysine • HCl	A	+	..	+	+
Methionine	+	+	+	+	+
Phenylalanine	+	+	+	+	+
Proline	+	+	+	+	+
Serine	+	+	+	+	+
Threonine	+	+	+	..	+
Tryptophan	—	C	D	—	G
Tyrosine	+	C	D	—	—
Valine	+	+	+	+	+

A—Some samples of L-arginine monohydrochloride and L- and DL-lysine monohydrochlorides give a white turbidity which, if too heavy, obscures the orange-red color formed from ammonia and Nessler's reagent.

B—In the presence of histidine no color is formed either with an excess of ammonia or Nessler's reagent.

C—Since both nitric acid and heating must be omitted, a positive test for iron indicates only ferrous ions.

D—0.5 N HNO<sub>3</sub> is substituted for 2 N HNO<sub>3</sub>, and observations are made in the minimum time to avoid the formation of brown or red solutions in which the white turbidities cannot be compared accurately.

E—If a gas is formed the supernatant yellow liquid should be decanted and replaced with distilled water; otherwise, the yellow precipitates cannot be compared accurately.

F—The blue-green supernatant liquid does not interfere in making accurate comparisons of the yellow precipitates.

G—A suspension of tryptophan in 1 ml. of 0.5 N HNO<sub>3</sub> is dissolved by heating it below 50°C. to prevent the formation of an interfering brown color.

placed in a desiccator over anhydrous calcium chloride and, after 30 minutes cooling, it is weighed at three one-minute intervals. The weights are plotted and the curve extrapolated to zero time. The heating and weighing procedures are repeated until the extrapolated weight is constant within 0.5 mg. An accurately weighed 1 to 1.5 g. sample of the amino acid is transferred from a weighing bottle to the crucible. The crucible is heated for 24 hours in a vacuum oven at 70°C. and 10 mm. The crucible is removed, cooled and weighed in the manner described. Moisture determinations may be made satisfactorily at 102°C. according to Chibnall *et al.* (151), and at 110°C. according to Brand and Kassell (122).

Moisture has been determined satisfactorily in alanine, arginine monohydrochloride, aspartic acid, cystine, glycine, histidine monohydrochloride monohydrate (the water of crystallization is not lost at 75°C. *in vacuo*), isoleucine, leucine, lysine monohydrochloride, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine. From 0.2 to 1.2% of volatile material have been found in arginine (free-base) dried at 60°C. and stored in a stoppered bottle. Moisture in glutamic acid is difficult to determine accurately owing to the ready dehydration of this amino acid to pyroglutamic acid. The determination of moisture appears to be reasonably accurate if glutamic acid is heated below 50°C. in an evacuated oven. Only air-dried samples of amino acids should be employed for moisture determination since oven-dried products are often too hygroscopic to be maintained moisture-free and to be weighed accurately.

b. *Ash.* In the determination of ash, a glazed porcelain crucible of approximately 25-ml. capacity is heated in a muffle furnace for 3 hours at 600°C. After the crucible has cooled to about 90°C. it is placed for 60 minutes in a desiccator over anhydrous calcium chloride. The crucible is weighed using the technique described in Section a. The heating and weighing procedures are repeated until the extrapolated weight is constant within 0.2 mg. An accurately weighed 1 to 1.5 g. sample of the amino acid is transferred from a weighing bottle to the crucible. The crucible is heated in the muffle furnace for 3 hours at 600°C. after which the crucible is cooled, and weighed in the manner described. Ash has been determined satisfactorily by this method in all of the amino acids listed in Table III except cysteine and 3,5-diiodo-tyrosine, which have not been tested. This method is essentially the same as that described by Chibnall *et al.* (151).

Warner (847) has recommended that calcium acetate be added to protein samples prior to ashing to bind phosphorus as a non-volatile

salt of calcium. Calcium acetate was added equivalent to the theoretical ash derived from phosphorus, and phosphorus was determined in the original sample by the method of Fiske and SubBarow (315). Although it was assumed that  $\text{Ca}_2\text{P}_2\text{O}_7$  was formed during the ashing process, it may be that  $\text{Ca}_3\text{P}_2\text{O}_8$  (657),  $\text{Ca}_3(\text{PO}_4)_2$  (437) or other salts of calcium and phosphorus would be formed under some conditions. This problem of proteins does not appear to be a critical one for highly purified amino acids which have been shown to contain less than 0.004% phosphorus.

c. *Semimicro-Kjeldahl Determination of Nitrogen.* Numerous modifications have been made in the procedure proposed in 1883 by Kjeldahl (465) for the determination of total nitrogen but, until recently, methods of proven reliability have not been available. Recoveries ranging from 98.2 to 100.73% were obtained by Miller and Houghton (571) who determined total nitrogen in twelve amino acids by special digestion and distillation procedures. By their standard procedure, the amino acid sample (0.5 to 1.5 mg.) is digested 6 hours after clearing with a mixture containing 500 mg. of potassium sulfate, 50 mg. of mercuric oxide and 1.5 ml. of concentrated sulfuric acid.

Of the amino acids alanine\*, arginine, cystine\*, glutamic acid\*, histidine, isoleucine\*, leucine\*, lysine\*, phenylalanine\*, tryptophan, tyrosine\* and valine\*, those marked with an asterisk have been analyzed in the writers' laboratory with an accuracy at least equivalent to that reported by Miller and Houghton. The apparatus, the digestion mixture (2 ml. of a sulfuric acid solution containing 5 mg. of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.5 mg. of powdered selenium and 250 mg. of  $\text{KHSO}_4$ ), and the experimental procedure were essentially the same as those described by Ma and Zuazaga (544). The samples of amino acid analyzed contained from 3-10 mg. of nitrogen. The additional amino acids hydroxyproline, and methionine were analyzed satisfactorily by this method.

It has been observed in the writers' laboratory that lysine may be determined quantitatively with the selenium-copper catalyst only after 50 hours digestion whereas only 26 hours were required in the absence of selenium. Using a sodium selenate catalyst, Chibnall *et al.* (151) found that 12 hours were adequate when the mixture was boiled briskly with the aid of an electric heater. Digestion for 4-5 hours with a  $\text{HgCl}_2$  catalyst was recommended by these authors. Although Van Slyke *et al.* (812) recovered a maximum of 90% nitrogen from lysine digested with a series of oxidizing and catalytic reagents, Weissman and Schoenheimer (852) reported good results after 2-hour digestion with a  $\text{HgSO}_4$  catalyst.

The observation of Vickery and Winternitz (830) that 8 hours digestion time are required for the quantitative determination of nitrogen

in histidine with a selenium catalyst was confirmed by Chibnall *et al.* (151). Van Slyke *et al.* (812) could not determine tryptophan quantitatively, and Jonnard (436) recovered only 86.5 to 97.3% of tryptophan nitrogen using a mixture of cupric sulfate, potassium sulfate and potassium persulfate as catalyst. The recoveries ranged from 95 to 101.7% using 57% HI, potassium sulfate, selenious oxide and cupric sulfate. Pepkowitz and Shive (630) obtained 101.5% of nitrogen after digesting tyrosine for 16 minutes with selenium oxychloride and perchloric acid. Alcock (52) has reported recently that nitrogen is liberated quantitatively from proteins (25 mg. nitrogen) which have been digested for 6 hours with 0.2 g. of cupric sulfate and 12.5 ml. of a 40% solution of sodium sulfate in concentrated sulfuric acid. It appears from this study that cupric sulfate is an effective catalyst at the elevated temperature of a boiling concentrated sulfuric acid solution containing 40% sodium sulfate.

*d. Van Slyke Nitrous Acid Determination of Amino Nitrogen.* The identity of an amino acid which reacts normally with nitrous acid may be determined with the aid of the Van Slyke (808) volumetric apparatus. Since analyses may be made only with a precision of 1 to 2%, this apparatus is not satisfactory for the determination of purity of amino acids. Since 0.0001 mg. of nitrogen per ml. of a 5-ml. sample produces a change in pressure of 1.3 mm., the Van Slyke (810) manometric apparatus, fitted with the Harington-Van Slyke (379-a) absorption chamber, is sufficiently sensitive for the quantitative determination of amino acids. Samples containing 0.5 to 1.0 mg. of amino nitrogen in 5 ml. of solution may be determined with a precision higher than 0.2%. Literature data on the determination of amino nitrogen in amino acids are given in the papers referred to in Table IV.

*e. Ninhydrin Analysis\*.* An amino acid containing adjacent free amino and free carboxyl groups reacts with ninhydrin to give carbon dioxide, ammonia and an aldehyde. The purity of such amino acids has been determined from the carbon dioxide and ammonia measured by manometric and titrimetric methods (155, 365, 546, 547, 693, 743, 811, 813, 814), and the amino acids may be determined conveniently and accu-

\* Hamilton and Van Slyke [*J. Biol. Chem.* 164, 249 (1946)] have confirmed the observation of Schott *et al.* (693) that the addition of hydrazine to the reagents in the gas chamber increases the accuracy by abolishing a plus error caused by volatile aldehydes formed in the determination of carboxyl nitrogen of valine and the leucines. Although these authors found that analyses of other amino acids were not affected by the aldehydes formed, and that the use of hydrazine lowered the results by less than 1% in determinations of free amino acids in biological materials, they have stated that the addition of hydrazine sulfate "used as reagent in the manometric method is recommended as a routine procedure."

TABLE IV

*Summary of Data on the Van Slyke Nitrous Acid Determination of Amino Acids*

Amino Acid	Volumetric Apparatus	References	Manometric Apparatus	References
Alanine	S	217, 684, 808*	S	455*
Arginine	A	808	A	211
Aspartic acid	S	808	S	*
Cystine	B	531, 684*	I	211, 455
Glutamic acid	S	684, 808*	S	455*
Glycine	C	684, 808*	J	211, 217, 329, 455
Histidine	S, D	808	S, D	329, 455
Isoleucine	S	*	S	*
Leucine	S	808*	S	211, 217, 810
Lysine	E	....	E	217, 455
Methionine	S	531	S	211*
Phenylalanine	S	684, 808*	S	329*
Serine	F	684, 808	S, K	211
Threonine	..	....	S	329, 815
Tryptophan	S, G	684, 808	S, L	211, 455
Tyrosine	S, H	317, 329, 684, 808*	M	329, 455*
Valine	S	808*	S	*

S—satisfactory analysis reported.

\*—satisfactory analysis obtained in writers' laboratory.

A—Nitrogen in excess of the normal (one mole of nitrogen per mole of arginine) is formed by reaction with nitrous acid at 45°C. (684) or for times longer than 4-5 minutes at 20 to 25°C. (411, 713, 812).

B—The abnormality, 105 to 112% (808) at 25°C., is increased at 45°C. (684). Approximately the theoretical nitrogen is liberated when the reaction mixture contains mercuric iodide (211).

C—Approximately 103% of the theoretical nitrogen is liberated at 25°C. (808), and the abnormality is greater at 45°C. (684).

D—Nitrogen greater than theoretical (one mole per mole of histidine) is liberated at 45°C. (684).

E—At 20°C., approximately one-half the total nitrogen (one mole of nitrogen per mole of lysine) is obtained (808). At 30°C. two moles of nitrogen are formed per mole of lysine in 15-20 minutes (217).

F—Nitrogen greater than the theoretical (one mole per mole of serine) is formed at 45°C. (684).

G—At 45°C., approximately two moles of nitrogen are formed per mole of tryptophan (684).

H—Results slightly higher than the theoretical have been reported (317, 329, 808). It has been found that the degree of abnormality increases as a function of the intensity of the illumination (317).

I—About 140% of the theoretical nitrogen is formed but in the presence of KI nearly the theoretical nitrogen is liberated (211, 455, 812).

J—In the presence of KI there is no abnormality, according to Kendrick and Hanke (455). Dunn and Porush (211) were unable to confirm this observation.



K—About 103% of the theoretical nitrogen is formed but in the presence of KI the nitrogen is nearly theoretical (211).

L—Nitrogen values higher than normal result at 45°C. (684) or from reaction times longer than 4-5 minutes at 20 to 25°C. (812). In the presence of KI, the nitrogen liberated is approximately 50% of the theoretical (one mole of nitrogen per mole of tryptophan) (211, 455, 812).

M—The nitrogen liberated is theoretical when the reaction is run for 15 minutes in the dark but values as high as 167% of normal have resulted from reactions run under strong illumination (329).

rately in quantities equivalent to 0.04 to 4.1 mg. of carboxyl nitrogen by the manometric carbon dioxide method of Van Slyke *et al.* (811). Modifications of this procedure have been described by MacFadyen (546), Hamilton and Van Slyke (365), Van Slyke *et al.* (814), and Schott *et al.* (693). Precision of 0.1 to 0.4% is readily attainable. The titrimetric carbon dioxide methods of Christensen *et al.* (155), and of Van Slyke *et al.* (813), and the titrimetric ammonia methods of MacFadyen (547), and Sobel *et al.* (743) are useful when a manometric apparatus is not available although the manometric analyses are more accurate. All of the amino acids may be determined with high accuracy although the conditions under which some of the amino acids, especially cystine and lysine, are determined must be controlled as stated in the footnotes to Table V.

f. *Glass Electrode Titration.* Since the  $pK_2$  values of aspartic acid and glutamic acid are 3.86 and 4.07, respectively (216), these amino acids may be titrated directly and accurately with standard base using a glass electrode to determine the end point. The other amino acids are too weak acids<sup>a</sup> ( $pK_2$ , 6.48 for 3,5-diiodotyrosine to 10.6 for proline) to be titrated accurately by this method. For this reason, the acid strength is increased about fourfold by the addition of formaldehyde to aqueous solutions of these amino acids. Under these conditions, the amino acids<sup>b</sup> may be titrated with precision and accuracy as high as 0.1% by the Dunn and Loshakoff (210) modification of the classical formol titration procedure of Sørensen (746). A highly sensitive glass-electrode apparatus is re-

<sup>a</sup>—The  $pK_3$  values for cystine (8.00), histidine (9.18) and lysine (10.5) correspond to the  $pK_2$  values of comparable dissociating groups of the other amino acids.

<sup>b</sup>—Histidine has not been analyzed by glass electrode titration although the analysis should present no special difficulty. No procedure has been found for the glass electrode titration of cystine owing to the low solubility of this amino acid in water and its slow rate of solution during the titration of its suspension with standard base. Although tyrosine is only slightly soluble in cold water, an aliquot which has been taken from a supersaturated solution prepared by dissolving this amino acid in boiling water, cooling the solution to room temperature and making it up to volume in a volumetric flask may be titrated successfully after the addition of formaldehyde if these manipulations are completed quickly.

TABLE V

*Summary of Data on Ninhydrin Determination of Amino Acids*

Amino Acid	References to Methods	Preferred Method		
		References	pH	Time* Minutes
Alanine <sup>a</sup>	155, 365, 547, 693, 743, 811, 813, 814	693	2.5	7
Arginine	546, 693, 743, 811	693, 811	2.5	8
Aspartic acid <sup>b</sup>	155, 546, 547, 693, 743, 811	811	2.5	6
		693	2.5	3
Cystine <sup>c</sup>	155, 693, 743, 811	811	1.0	8
		693	4.7	7.5
Glutamic acid	155, 547, 693, 743, 811	811	2.5	7
		693	2.1	3
Glycine <sup>d</sup>	155, 365, 547, 693, 811	811	4.7	6
Histidine	547, 693, 743, 811	811	2.5	8
		693	2.5	2
Hydroxyproline <sup>e</sup>	693, 811	811	2.5	7
		693	2.5	2
Isoleucine <sup>f</sup>	693	693	2.5	7
Leucine <sup>f</sup>	155, 693, 743, 811	693	2.5	5
Lysine <sup>g</sup>	155, 365, 693, 743, 811	811	1.0	8
Methionine <sup>h</sup>	693, 743, 811	811	1.0	8
		811	2.5	7
Phenylalanine <sup>i</sup>	155, 693, 743, 811	693	2.5	3
		811	2.5	7
Proline	693, 811	693	2.5	4
		811	2.5	7
Serine	155, 693, 743, 811	693	2.5	5
		811	2.5	7
Threonine <sup>j</sup>	693, 743, 811	693	2.5	4
		693, 811	4.7	7
Tryptophan <sup>k</sup>	155, 693, 547, 743, 811	811	2.5	7
Tyrosine <sup>m</sup>	155, 693, 743, 811	693	2.5	2
		693	2.5	10
Valine <sup>f</sup>	155, 693, 743, 811	693	2.5	10

\*—Number of minutes given is minimum time required for quantitative results. Longer times up to 30 minutes are equally satisfactory except for the amino acids referred to in the footnotes.

<sup>a</sup>—MacFadyen (547) obtained 90%, and Sobel *et al.* (743) the theoretical quantity of ammonia. The manometric carbon dioxide procedure yields theoretical results with salt-free reagents (811) while the high value (102%) obtained with salt-saturated reagents may be reduced to the theoretical with hydrazine (693).

<sup>b</sup>—Two moles of CO<sub>2</sub> are formed per mole of aspartic acid.

<sup>c</sup>—The results with cystine are variable under different experimental conditions. It has been determined from rate studies made by Van Slyke *et al.* (811) and Schott *et al.* (693) that theoretical results may be obtained in 8 minutes at pH 1 using salt-free reagents, in 7.5 minutes at pH 4.7 using salt-saturated reagents, and in 2 minutes at pH 2.5 and in 5 minutes at pH 1 using salt-saturated reagents and

the hydrazine technique. Christensen *et al.* (155) found 96.2% of the theoretical amount of carbon dioxide by a titrimetric carbon dioxide method for a sample of cystine which contained 96.7% of the theoretical nitrogen by Kjeldahl analysis while Sobel *et al.* (743) obtained only 73% of the theoretical nitrogen by their ammonia method.

<sup>d</sup>—In salt-saturated reagents, 97.5% of the theoretical nitrogen was obtained either with or without the hydrazine technique (693). The titrimetric-carbon dioxide method yields only 95% of the theoretical  $\text{CO}_2$  in 15 minutes at 110–115°C. (155). MacFadyen (547) found 86% of the theoretical nitrogen in 8 minutes at pH 2.5 by his ammonia method while Sobel *et al.* (743) obtained 100.2% of ammonia nitrogen in 10 minutes at pH 2.5 and 100°C.

\*—Hydroxyproline yields no ammonia by MacFadyen's (547) procedure and only about 30% of the theoretical amount by the modified method of Sobel *et al.* (743).

<sup>f</sup>—The high values for isoleucine (105.4%), leucine (104.6%) and valine (104.4%) with salt-free reagents and for isoleucine (113.7%), leucine (114.5%) and valine (112.2%) with salt-saturated reagents are reduced to the theoretical amounts by the double-equilibration or the hydrazine technique (693).

<sup>g</sup>—The excess of theoretical carbon dioxide given by lysine increases with unit time as the pH is raised from 1 to 4.7 (693, 811) although the results are theoretical in 8.6 minutes at 100°C. and pH 1. By the alternate procedure of Van Slyke *et al.* (811), the reaction is allowed to proceed for a longer standard time (20 minutes), during which the initial reaction rate has slowed appreciably, and a correction factor of 2.5% is applied. Christensen *et al.* (155) observed that a sample of DL-lysine dihydrochloride containing 116.5% of the theoretical nitrogen gave 112.5% of the theoretical  $\text{CO}_2$ , measured titrimetrically, in 15 minutes at 110–115°C. MacFadyen (547) obtained 101% of the theoretical  $\text{CO}_2$  from lysine dihydrochloride in 10 minutes at pH 1 by his manometric procedure. The results were quantitative in 10 minutes at pH 1 by his ammonia procedure but they were 103% when the time was extended to 30 minutes. Sobel *et al.* (743) found 99.5% of the theoretical nitrogen in 12 minutes at pH 2.5 by their ammonia method.

<sup>h</sup>—The salt-free reagent appears to be preferable for the determination of methionine since values about 0.5% above the theoretical were found with salt-saturated reagents both at pH 2.5 and 4.7 (693). The ammonia procedure of Sobel *et al.* (743) yields only 96% of the theoretical nitrogen.

<sup>i</sup>—Phenylalanine gives only 97% of the theoretical nitrogen by the ammonia method of Sobel *et al.* (743).

<sup>j</sup>—Threonine yields 98.2% of ammonia nitrogen by the procedure of Sobel *et al.* (743).

<sup>k</sup>—Low values for tryptophan result at pH 1, 2 and 2.5 (693, 811). Quantitative data were obtained by Christensen *et al.* (155) in 15 minutes at 110–115°C. by the titrimetric- $\text{CO}_2$  procedure. The ammonia methods of MacFadyen (547) and Sobel *et al.* (743) yielded only 34% and 50%, respectively, of the theoretical nitrogen.

<sup>m</sup>—The ammonia method of Sobel *et al.* (743) gave only 97.6% of the theoretical nitrogen.

quired for the determination of amino acids by this method. The Robertson (672) apparatus, which has been employed with some modifications in the writers' laboratory, and its application to the analysis

of amino acids have been discussed by Dole (195). Technical difficulties in the preparation, aging and maintenance of thin, glass-bulb electrodes of the Robertson type have been a barrier to its use.

In titration of amino acids by this method, it is desirable on theoretical grounds to employ, especially in the proximity of the end point, increments of standard base which are equal and as small as possible although it has been the practice to use increments of 0.25 *N* base varying from 0.02 to 0.20 ml.

*g. Specific Rotation.* The specific rotation is a reliable index of purity of the optically active forms of the amino acids for which standard values have been established. Literature data considered to be the most dependable have been listed by Dunn *et al.* (216) although some of these specific rotations are inaccurate because of the uncertain purity of the sample analyzed, low precision of the observed rotation and for other reasons. Specific rotations of most of the amino acids have been reported by numerous investigators but all of the factors which determine the magnitude of the values have been well controlled only in a few cases. The data reported for L-leucine (759), L-histidine (209) and L- and D-alanine (225) are considered to be in the latter category.

Specific rotations which are thoroughly satisfactory as standards in determining the degree of purity of amino acids should be accurate within a limiting error of 0.1 to 0.2%. The most critical variables are purity and concentration of the solute and the solvent, the temperature, and the precision of the observed optical rotation. Some of these variables may be readily controlled but an amino acid solute may be prepared and proved pure only by laborious procedures of the type employed in the writers' laboratory (209, 225). An amino acid is considered arbitrarily to be pure if, within the limits of a small probable error, it is free from the most probable inorganic impurities, if it contains the theoretical percentage of total nitrogen, amino nitrogen or carboxyl, if it (limited to optically active forms) has a rotation which undergoes no change on recrystallization of the sample, and if it has been shown by differential solubility measurements to contain only one chemical entity. A discussion of the latter point is given in the following section on solubility. Observed optical rotations of acceptable precision may be obtained by an experienced worker with the aid of a sensitive ( $0.01^\circ$ ) polarimeter provided the concentration of solute, type and concentration of solvent, length of the tube and other experimental factors can be and are regulated to give an observed rotation of about  $5.00^\circ$  or higher value. Although this "ideal" has been attained only in a few cases (see Table VI), it should be possible with other amino acids to measure rotations

under comparable conditions. Low solubilities in any common solvent, intrinsically low rotations under any conditions, and relative insensitivity of available polarimeters are immediate barriers to the accurate determination of specific rotations of some amino acids. In such cases a polarimeter of the type described by Levene *et al.* (509) would be useful since mean values of duplicate series of readings, agreeing within  $0.001^\circ$ , are obtainable with this instrument.

A summary of the specific rotations of the amino acids and the experimental conditions which, at the present time, are considered to be as satisfactory as any other for the measurement of rotation is given in Table VI. Only values for the L-antipodes are listed since those for

TABLE VI  
*Specific Rotations of the Amino Acids\**

Amino Acid	c	Solvent	l	Temp. °C.	$\alpha$	$[\alpha]_D$	Reference Numbers
Alanine	10.03	5.97 N HCl	4.000	25.0	5.805	14.47	225
Arginine	....	6.0 N HCl	4.000	23.3	1.825	27.58	a
Aspartic acid	....	6.0 N HCl	4.000	24.0	1.972	24.62	b
Cystine	0.9974	1.02 N HCl	2	24.91	-4.242	-212.9	739
3, 5-Diiodotyrosine	5.08	1.1 N HCl	1	20	0.15	2.9	14
Glutamic acid	7.008	1.73 N HCl	4.000	25.0	8.888	31.71	c
Histidine	3.770	Water	4.000	24.90	-5.904	-39.20	209
Hydroxyproline	....	Water	4.000	22.5	-3.009	-75.2	b
Isoleucine	....	6.1 N HCl	1	20	2.07	40.6	524
Leucine	9.075	4.5 N HCl	4.000	25.0	5.050	13.91	b
Lysine	1.642	6.08 N HCl	4.000	25.0	1.729	25.72	b
Methionine	5	3 N HCl	2	20		23.4	229
Phenylalanine	1.936	Water	2	20	-1.36	-35.1	307
Proline	0.9998	Water	4.000	25.3	-3.414	-85.0	d
Serine	9.344	1 N HCl	1	25	1.35	14.5	299
Threonine	....	Water	2	26	-0.625	-28.3	855
Thyroxine	....	0.13 N NaOH in 70 % ethanol	1	...	-0.147	-4.4	322
Tryptophan	2.071	Water	4.000	25.0	-2.663	-32.15	b
Tyrosine	4.005	6.08 N HCl	4.000	25.0	-1.165	-7.27	a
Valine	3.4	6.0 N HCl	2	20	1.93	28.8	299

\*—Specific rotations of different samples can be compared accurately only under identical conditions. Some studies have been made by Lutz and Jirgensons (535, 536) of the rotations of the amino acids in solutions of varying pH and the temperature coefficients for aspartic acid, cystine, glutamic acid, histidine, leucine and tyrosine have been listed by Dunn *et al.* (209). The latter authors have investigated the specific rotation of L-histidine in water as a function of concentration. More extensive information is needed relating specific rotations of the amino acids to these and other variables.

c—grams of solute (calculated as free-base amino acid) per 100 ml. of solvent.

l—length of tube in decimeters.

$\alpha$ —observed rotation in angular degrees.

D—sodium light (5893 Å).

a—unpublished data by L. B. Rockland in the writers' laboratory.

b—unpublished data by M. P. Stoddard in the writers' laboratory.

c—unpublished data by R. C. Bovie in the writers' laboratory.

d—unpublished data by G. W. Courtney in the writers' laboratory.

the optical isomers would be of equal magnitude although of opposite sign. The data selected have been limited with one exception to values in water and HCl since rotations in any alkaline solution might be inaccurate owing to racemization or partial degradation of the amino acid.

*h. Solubility.* The purity of an amino acid may be determined accurately by solubility measurements in those cases where accurate solubility data are available. A wealth of carefully determined solubility values for nearly all of the amino acids, but not for all of the L- and DL-forms, has been reported by Dalton and Schmidt (185, 186), Dunn *et al.* (214, 215), and some other workers. Any of these data undoubtedly are of relatively high accuracy, since the samples analyzed were subjected to drastic purification procedures, yet some of the values probably are somewhat inaccurate owing to uncertainty concerning the effectiveness of the purification procedures. It seems desirable, therefore, that new solubility studies be carried out especially on cystine, glutamic acid, threonine, proline, hydroxyproline, methionine and isoleucine using samples and conditions which would reduce the probable errors inherent in the values which have been reported.

Differential (Phase Rule) solubility principles have been applied extensively to the determination of purity of purified proteins (486, 592) but only in a few cases to amino acids (209, 225). In order to determine the purity of an amino acid by the differential solubility method, minimum requisites are solubility values determined in duplicate on at least two levels of solute in excess of the solubility amount. The attainment of solubility equilibrium should be established on the basis of the constancy of the solubility data at each level of solute either by measuring solubilities of samples brought initially to a temperature below and one above that of the thermostat or equilibrated for increasing times.

If it is assumed that each component of a two-component system has independent solubility and that the solubility values  $Y_1$  at  $X_1$  excess of solute,  $Y_2$  at  $X_2$  excess of solute and  $Y_3$  at  $X_4$  and  $X_5$  excesses of solute were found, these data would be represented graphically by the solid straight lines shown in Fig. 1. It is apparent from Fig. 1 that  $Y_0$  is the solubility of the amino acid, that  $Y_3$  is the sum of the solubilities of the amino acid and the impurity and that  $Y_3 - Y_0$  is the solubility of the impurity. It follows, therefore, that:

$M$ , the fraction of the impurity in the amino acid sample, is the slope,  $(Y_2 - Y_1)/(X_2 - X_1)$ , of the curve,  $Y_0 Z$ .

$Y_1$ , the observed solubility at  $X_1$ , is equal to  $MX_1 + Y_0$ .

$Y_2$ , the observed solubility at  $X_2$ , is equal to  $MX_2 + Y_0$ .

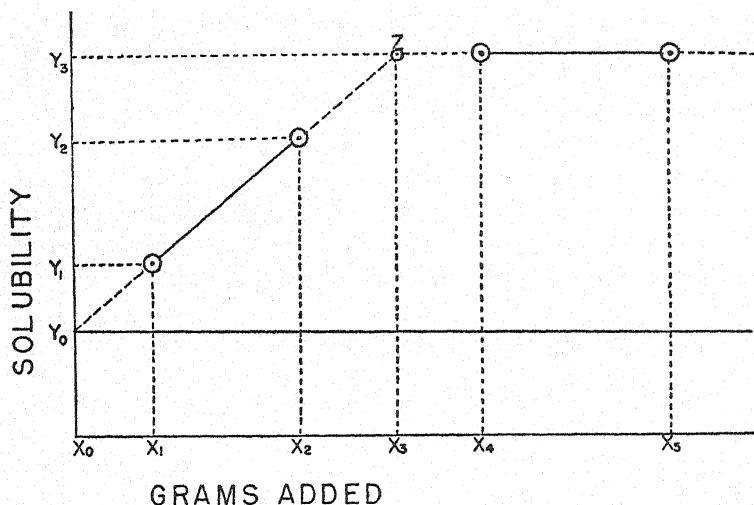


Fig. 1. Phase rule solubility diagram.

$Y_0$ , the solubility value for the amino acid, is equal to  $Y_1 - MX_1$  and to  $Y_2 - MX_2$ .

$P$ , the percentage purity of the amino acid, is equal to  $100 - M100$ .

The literature data considered to be most reliable for the solubilities of the amino acids in water at various temperatures are listed in Table VII.

*i. Microbiological Assay.* Traces well below 0.1% of an impurity of almost any amino acid in a sample of another amino acid may be determined conveniently and with relatively high accuracy by microbiological assay with one or more of the six lactic bacteria listed in Table VIII. Although microbiological procedures have been developed only recently they have become one of the most useful tools for the determination of purity of amino acids. It is noteworthy that the degree of purity of most amino acids required as standards in the microbiological assay of amino acids can be determined with sufficient accuracy for this purpose only by microbiological assay.

Various amino acids have been detected as impurities in available samples of other amino acids by several investigators. Hegsted and Wardwell (385) found isoleucine as a contaminant in five of seven samples of DL-leucine examined while Baumgarten *et al.* (78) observed that a sample of L-leucine contained isoleucine and methionine and that one of L-tryptophan contained tyrosine. In the writers' laboratory, microbiological assay methods have been used extensively to determine various amino acid impurities in amino acids in process of purification as well as

TABLE VII

*Solubilities of Amino Acids in Grams Per 100 Grams of Water*

Amino Acid	Temperature, ° C.					Reference Numbers
	0°	25°	50°	75°	100°	
DL-Alanine	12.11	16.72	23.09	31.89	44.04	185
L-Alanine	12.73	16.51 <sup>a</sup>	21.79	28.51	37.30	185
D-Alanine	...	16.49 <sup>a</sup>	...	...	...	...
DL-Aspartic acid	0.262	0.778	2.000	4.456	8.594	185
L-Aspartic acid	0.209	0.500	1.199	2.875	6.893	185
DL-Cystine × 10 <sup>2</sup>	...	0.49 <sup>b</sup>	...	...	...	...
L-Cystine × 10 <sup>2</sup>	0.502	1.096	2.394	5.229	11.42	186
D-Cystine × 10 <sup>2</sup>	...	1.08 <sup>b</sup>	...	...	...	...
meso-Cystine × 10 <sup>2</sup>	...	0.56 <sup>b</sup>	...	...	...	...
DL-Diiodotyrosine × 10	0.149	0.340	0.773	...	...	869
L-Diiodotyrosine × 10	0.204	0.617	1.862	5.62	17.00	185
DL-Glutamic acid	0.855	2.054	4.934	11.86	28.49	185
L-Glutamic acid	0.341	0.843 <sup>c</sup>	2.186	5.532	14.00	185
Glycine	14.18	24.99	39.10	54.39	67.17	185
L-Histidine	...	4.29	...	...	...	209
L-Hydroxyproline	28.86	36.11	45.18	51.67 <sup>f</sup>	...	791
DL-Isoleucine	1.826	2.229	3.034	4.607	7.802	185
L-Isoleucine	3.791	4.117	4.818	6.076	8.255	186
DL-Leucine	0.797	0.991	1.406	2.276	4.206	185
L-Leucine	2.270	2.19 <sup>d</sup>	2.66 <sup>e</sup>	3.823	5.638	185
D-Leucine	...	...	2.66 <sup>e</sup>	...	...	...
DL-Methionine	1.818	3.381	6.070	10.52	17.60	186
DL-Phenylalanine	0.997	1.411	2.187	3.708	6.886	185
L-Phenylalanine	1.983	2.965	4.431	6.624	9.900	186
L-Proline × 10 <sup>-1</sup>	12.74	16.23	20.67	23.90	...	186
DL-Serine	2.204	5.023	10.34	19.21	32.24	186
L-Tryptophan	0.823	1.136	1.706	2.795	4.987	186
DL-Tyrosine × 10	0.147	0.351	0.836	...	...	869
L-Tyrosine × 10	0.196	0.453	1.052	2.438	5.650	185
D-Tyrosine × 10	0.196	0.453	1.052	...	...	869
DL-Valine	5.98	7.09	9.11	12.61	18.81	185
L-Valine	8.34	8.85	9.62	10.24 <sup>f</sup>	...	187

<sup>a</sup>—Dunn *et al.* (225).<sup>b</sup>—Loring and du Vigneaud (530).<sup>c</sup>—Unpublished data by R. C. Bovie in the writers' laboratory.<sup>d</sup>—Stoddard and Dunn (759).<sup>e</sup>—Fox (326).<sup>f</sup>—Value at 65°C.

to insure the purity of the amino acid samples employed as standards in microbiological assay investigations. References to the literature on microbiological methods for the determination of the amino acids with lactic acid bacteria are given in Table VIII. Microbiological procedures



TABLE VIII

*Literature References to Microbiological Methods  
For Determination of Amino Acids*

Amino Acid	<i>L. arabinosus</i> 17-5	<i>L. casei</i>	<i>S. faecalis</i> ( <i>S. lactis</i> R)	<i>L.</i> <i>mesenteroides</i> P-60	<i>L.</i> <i>fermenti</i> 38	<i>L.</i> <i>pentosus</i>
Alanine	.....	.....	123	.....	.....	.....
Arginine	.....	76, 78, 397, 398, 543, 717, 735, 760	357, 402, 762	.....	.....	.....
Aspartic acid	.....	.....	735	72, 123, 362	.....	.....
Cystine	76, 716	.....	.....	.....	.....	.....
Glutamic acid	76, 78, 123, 205, 383, 398, 514, 537, 716	76, 78, 363, 717	77	363	.....	363
Glycine	.....	.....	.....	123	.....	.....
Histidine	.....	.....	78, 357, 762	207, 213, 397, 398, 402, 735	220	.....
Isoleucine	76, 78, 123, 384, 396-398, 402, 485, 711, 716	76, 78	762	.....	.....	.....
Leucine	76, 78, 154, 384, 385, 396-398, 485, 710, 711, 716	76, 78, 717, 760	402, 762	.....	.....	.....
Lysine	.....	.....	357, 735, 762	72, 123, 397, 398, 402, 735	.....	.....
Methionine	206, 716, 760	.....	78, 762	72, 206, 402	206	.....
Phenylalanine	123, 384	76, 78, 219, 397, 398, 717, 760	.....	72, 219, 402	.....	.....
Proline	.....	.....	.....	72	.....	.....
Serine	.....	76	.....	.....	.....	.....
Threonine	76, 78, 396- 398, 716, 760	.....	78, 355, 402, 762	.....	221	.....
Tryptophan	76, 154, 218, 352, 353, 397, 402, 716, 734, 882, 883	76, 717, 734, 760	734, 762	734	.....	734
Tyrosine	.....	76, 78, 397, 398, 717	.....	402	.....	.....
Valine	76, 78, 123, 154, 384, 396, 398, 402, 485, 543, 710, 716	76, 78, 543, 717	357, 762	.....	.....	.....

for the determination of amino acids with molds and other types of organisms have not been considered. A general review of the microbiological assay of amino acids has been given by Snell in Volume II of "*Advances in Protein Chemistry*."

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<sup>1</sup> DL- $\alpha$ -Bromopropionic acid may be prepared from propionic acid and bromine in 80-90% yield by the method of Heidelberg (386).

<sup>2</sup> A tank containing 30 pounds of anhydrous hydrocyanic acid may be purchased from the American Cyanamid Company, El Monte, California. Any desired quantity of liquid hydrocyanic acid may easily be obtained by passing gas from the tank through a condenser cooled with ice water and collecting the liquid in a graduate immersed in an ice-water bath. These manipulations should be carried out in a hood or on an open porch and the worker should wear an efficient gas mask. Although the vapors of hydrogen cyanide are less toxic than those of hydrogen sulfide, safe handling of the former gas is more difficult because of the absence of any pronounced odor.

<sup>3</sup> Commercial Solvents Corporation.

<sup>4</sup> Prepared by the method of Doherty *et al.* (194) and purified as described by Stein *et al.* (755).

<sup>5</sup> Prepared by the method of Griess (356) as modified by Stein *et al.* (755).

<sup>6</sup> Prepared by Bergmann's (86) modification of Meisenheimer's (567) method.

<sup>7</sup> Prepared by acidifying a solution of commercial sodium flavianate (Naphthol Yellow S, National Aniline and Chemical Company).

<sup>8</sup> Ammonium hydroxide and ethanolamine have been found more satisfactory than pyridine or aniline in the writers' laboratory.

<sup>9</sup> Prepared from fumaric acid by the method of Corson *et al.* (173, 552).

<sup>10</sup> Aspartic acid may be isolated directly in lower yield without initial precipitation as the copper salt.

<sup>11</sup> *Lupinus albus* seeds have not been available commercially since 1942. *Lupinus angustifolius* seeds have been obtainable from F. H. Woodruff and Sons, Milford, Connecticut, as well as from concerns in Florida where the blue lupine (*L. angustifolius*) is grown as a cover crop. According to information received from the University of Florida Agricultural Experiment Station (Gainesville) through the courtesy of Dr. W. E. Stokes, seeds harvested in April germinate usually 90% or better if they have been properly harvested and stored.

<sup>12</sup> The testa (seed covers) usually drop off during sprouting.

<sup>13</sup> Experiments by N. Juster and L. E. McClure in the writers' laboratory.

<sup>14</sup> Piutti (636) prepared 20 kg. of asparagine from 6500 kg. of vetch seedlings.

<sup>15</sup> Prepared from benzyl mercaptan and polyoxymethylene by the method of Böhme (113).

<sup>16</sup> L-Cystine was isolated from a urinary calculus in 1810 but its presence in proteins, first reported by E. Külz in 1886, was confirmed by Möerner (574, 575) in 1899. Vickery and Schmidt (829) have reviewed this topic.

<sup>17</sup> Prepared by the method of Snyder and Smith (741). The reactions are: malonic ester  $\rightarrow$  isonitrosomalonic ester  $\rightarrow$  aminomalonic ester  $\rightarrow$  diethyl acetamidomalonic ester.

<sup>18</sup> Reviews have been given by O'Day and Bartow (595, 596), Eldridge (243), Benninghoff (81), Albrook (51), Lyon (538) and Barta (71).

<sup>19</sup> The isolation of L-glutamic acid by precipitation, first as the ethanol-insoluble

calcium salt, and then as the hydrochloride according to Foreman's (318) method, has been employed primarily for analytical purposes.

<sup>20</sup> Prepared by the method of Cerchez and Colesiu (145) and Tullar (801).

<sup>21</sup> Prepared from glycerol- $\alpha,\gamma$ -dichlorohydrin and NaOH by the method of Clarke and Hartman (158).

<sup>22</sup> The  $\alpha$ -bromo- $\delta$ -chloro derivative described by Traube and Lehmann (798, 799) was used in the original synthesis.

<sup>23</sup> May be prepared satisfactorily from 2-methyl butanol through the intermediates, 2-methylbutyl bromide, 2-methylbutyronitrile and 3-methyl-n-valeric acid by the method of Hass and Marshall (382).

<sup>24</sup> Prepared in 55% yield by Fischer and Schmitz (306) from malonic ester through the intermediates, isobutylmalonic ester and isobutylbromomalonic acid. The latter substances were prepared essentially by the methods of Guthzeit (360) and Bischoff (101).

<sup>25</sup> May be prepared satisfactorily from isobutanol through the intermediates, isobutyl bromide and isobutyronitrile by the method of Hass and Marshall (382).

<sup>26</sup> Essentially the method of Locquin and Cerchez (525, 526).

<sup>27</sup> It has been found by M. P. Stoddard in the writers' laboratory that L-leucine is racemized rapidly by boiling glacial acetic acid.

<sup>28</sup> The preparation of L-methionine from the mother liquors has been carried out successfully in the writers' laboratory.

<sup>29</sup> The oxime is most conveniently prepared from cyclohexanone and commercially available hydroxylamine sulfate which may be used without purification.

<sup>30</sup> It has been found in the writers' laboratory that a mixture containing 120 g. of cyclohexanoneoxime and 30 N  $\text{H}_2\text{SO}_4$  (2 cc. per g. of oxime) may be rearranged smoothly and without violence by heating it with a small flame until a visible reaction starts at the point of heating. The conditions employed by Eck and Marvel were those utilized by Wallach (844) and Ruzicka (675).

<sup>31</sup> See Block and Bolling (111) for discussion of methods and references to the literature.

<sup>32</sup> Prepared from  $\beta$ -hydroxymethyl sulfide and thionyl chloride (463) essentially by the method of Kirner (462).  $\beta$ -hydroxymethyl sulfide is prepared from methyl isothiourrea sulfate through methyl mercaptan and sodium methyl sulfide and reaction of the latter with ethylene chlorohydrin by the method of Windus and Shildneck (868). Methyl mercaptan is available commercially.

<sup>33</sup> Acetobutyrolactone is prepared by the method of Knunyantz (472).

<sup>34</sup> Prepared by Livak *et al.* (521) from  $\gamma$ -butyrolactone in 36% over-all yield through the intermediates,  $\alpha$ -bromo- $\gamma$ -butyrolactone and  $\alpha$ -amino- $\gamma$ -butyrolactone hydrobromide.

<sup>35</sup> Prepared from phenoxyethyl bromide (559) and malonic ester by the procedure of Fischer and Blumenthal (293).  $\alpha$ -Amino- $\gamma$ -butyrolactone hydrochloride has been prepared from  $\gamma$ -butyrolactone through the  $\alpha$ -bromo- $\gamma$ -butyrolactone by Livak *et al.* (521), by reduction of  $\alpha$ -oximino- $\gamma$ -butyrolactone with tin and HCl (450) or with Raney Nickel and hydrogen (450). The intermediate  $\alpha$ -acetamino- $\gamma$ -butyrolactone was hydrolyzed by the method of Hill and Robson (400).

<sup>36</sup> Methionine anhydride has been prepared by Snyder and Chiddix (738) from sodium methyl mercaptide and 3,6-bis-( $\beta$ -chloroethyl)-2,5-diketopiperazine.

<sup>37</sup> Quantity such that the ratio of mercury in the mercuric acetate to the methionine in the residual solid is 5 to 1. Toennies and Kolb (788) have found that

this ratio is optimum for the precipitation of methionine by  $\text{HgCl}_2$  or by mercuric acetate in the presence of chloride ions.

<sup>38</sup> The yield may be increased to about 80% with 100% excess of diethyl malonate (497).

<sup>39</sup> The reduction procedure is essentially the same as that described by Harington and McCartney (373) and Lamb and Robson (491).

<sup>40</sup> Snyder *et al.* (740) prepared N-acetyl DL-phenylalanine essentially by the same procedure.

<sup>41</sup> Prepared from ethyl glycinate by Fischer's (288) method.

<sup>42</sup> Yield is increased from about 27 to 38% with excess diethyl malonate (497).

<sup>43</sup> Prepared from dimethylamine, glacial acetic acid, formaldehyde and indole by the method of Kuhn and Stein (484) as modified by Snyder *et al.* (742). Gramine may be obtained from the Winthrop Chemical Company, Rensselaer, New York (148).

<sup>44</sup> Simultaneous hydrolysis and amination of (B) with ammonia have been found satisfactory in the writers' laboratory.

<sup>45</sup> Prepared by Snyder *et al.* (742) from sodium diethyl malonate and gramine methiodide.

<sup>46</sup> Prepared by Spielman (751) from 3-indoleglyoxylic acid, hydantoin, dimethylaniline and morpholine.

<sup>47</sup> Prepared from ethyl glycinate hydrochloride and NaOH by Fischer's (287) method.

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# The Plasma Proteins and Their Fractionation

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## I. INTRODUCTORY CONSIDERATIONS: FUNCTIONS OF PLASMA

For many purposes, blood may be regarded as a system containing a very great number of components, but composed of only two phases—the plasma, and the interior of the red cells. Such a conceptual scheme is, of necessity, an abstraction, far simpler than the concrete reality. It ignores the various types of white cells and their functions, which are of major importance to the biologist and the physician; it neglects such other structures as platelets. It ignores also the important and distinctive properties of the membrane which separates the plasma phase from the interior of the red cell. Nevertheless, even such a simplified model is sufficiently complex to require a very elaborate description in terms of chemical and physicochemical analysis, and it is adequate to describe a great number of the manifold biological functions of blood. Foremost among these must be placed the respiratory function, since even a very brief interruption of this means death to the organism. This function, involving the exchange of oxygen and carbon dioxide between blood, lungs and tissues, is primarily concerned with the red cell phase. In this connection the plasma acts as an indispensable transporting medium for the red cells, and the plasma proteins function as buffers in the maintenance of the pH stability of the system, although in this respect they play a much smaller role than the hemoglobin of the red cells.

This review will not be directly concerned with the respiratory function of the blood, which has received repeated and profound discussion by

many other workers. The classical monograph of L. J. Henderson (96) in 1928 formulated with comprehensive insight the nature of the fundamental equilibria involved and the complex interactions of the components of the system. Since 1928, the major advances in this field have been concerned with a more detailed knowledge of some of the mechanisms involved in the transport of carbon dioxide, and particularly with the kinetics of the reactions involved in oxygen and carbon dioxide transport. The discovery of the enzyme carbonic anhydrase has profoundly altered some of our conceptions concerning the reactions involving carbon dioxide. These advances have been well reviewed by Roughton (178, 179). The acid-base equilibria in hemoglobin, and their relations to its oxygen affinity, have also been studied more deeply and thoroughly in recent years (Wyman, 78, 220, 221).

In this review, we shall be concerned primarily with the distinctive properties and functions of the plasma, and especially of the plasma proteins, and only incidentally with the relations between plasma and cells. The functions of blood plasma itself are so many and so important that they cannot be exhausted by any brief list. However, it may clarify the later discussion somewhat if some of the most outstanding of these functions are briefly set forth here.

### 1. *Plasma in Maintenance and Stabilization of Blood Volume*

Since blood, in order to perform any of its other functions, must be a rapidly circulating medium, it is essential that the volume of the blood, and such physical characteristics as its viscosity, should be suitable for maintaining that circulation. If the volume of the plasma falls, a double strain is put on the heart: first, to maintain flow with an inadequate volume for pumping and maintaining pressure; second, to maintain this flow in the presence of the increased viscous resistance due to the increased concentration of red cells relative to plasma.\*

\*The term "viscosity" is used in this paragraph in a somewhat broad sense to indicate factors which produce increased resistance to flow through the capillaries. Since the diameter of the capillaries is of the same order of magnitude as that of the red cells themselves, and since the red cells are frequently in contact with the capillary walls during the flow (see Krogh, 120), it is clear that the conditions of flow cannot be at all comparable to those existing in the laminar flow of a pure viscous liquid in a capillary tube.

It is of some interest to note that the beautiful researches of Poiseuille on the viscous flow of liquids were inspired by his interest in the problem of the flow of blood through the capillaries. Later work has revealed, however, that blood, primarily because of the red cells in it, is one of the systems which does not obey Poiseuille's law of flow.

As was revealed by the original researches of Bayliss and Starling, the exchange of fluid between blood and tissues and the maintenance of blood volume is dependent on the balance between the hydrostatic pressure of the blood in the capillaries, tending to expel liquid from the blood into the tissues, and the osmotic pressure, due to the plasma proteins, which tends to draw liquid from the tissue spaces back into the blood. This osmotic function of the plasma proteins is largely determined by the serum albumin which, because of its relatively low molecular weight and its high net negative charge at the pH of blood, is far more effective than the same weight of the other plasma proteins would be in maintaining osmotic pressure. Albumin is also particularly fitted for this function because of the relatively low asymmetry of the molecule, which departs little from the spherical shape, and the consequent low viscosity of its solutions.

### *2. Transport of Hormones Between Tissues*

The interchange of hormones between one tissue and another is of necessity effected through the blood, and the plasma is the great highway of travel for these materials from the tissue where they are produced to those other tissues where their action is primarily exerted. Often, the hormones are present in blood in extremely low concentration so that it may be difficult or impossible to demonstrate the existence of a particular hormone by the assay of whole plasma. In some cases, however, a hormone may become readily demonstrable when concentrated in a particular plasma fraction, after suitable fractionation procedures. Such a result has indeed been demonstrated, for instance, by Professor F. L. Hisaw for the thyrotropic hormone, in plasma fractions obtained by the techniques later discussed in Part VI.

### *3. Transport and Mobilization of Antibodies*

The relation of antibodies to blood plasma is again somewhat similar to that of the hormones. They are produced in the tissues, but may exert their protective action either in another tissue or in the plasma itself, when they are brought into contact with the antigens for which they are specific. It would be arbitrary and unnecessary to discriminate in this respect between what might be called normal and pathological plasma. In any normal human or animal population, many if not most individuals will have developed antibodies to the antigens of certain bacteria and viruses. It is therefore natural to consider the antibodies present in significant concentration in the blood of mixed pools from normal individuals as among the normal components of plasma in the population. The inherent interest of the chemical nature and reaction

of these antibodies and the great practical importance associated with their use in immunization against certain diseases, certainly justify their consideration in this fashion.

#### 4. *Protection Against Blood Loss by the Clotting Mechanism*

Alteration of blood volume may, of course, occur not only by loss of plasma into the tissue spaces or externally, but by loss of whole blood from a wound. The mechanism of protection against such loss by clot formation has been known to man throughout his history, but the nature of the underlying chemical reactions is still very incompletely understood. However, a number of components have now been recognized as playing specific parts in the coagulation mechanism. Fibrinogen, which forms the main substance of the clot, has now been obtained in highly purified form, and prothrombin of bovine blood has also been prepared apparently as a pure protein.

Another component of normal blood is a certain small fraction of the globulin which appears to be lacking in cases of hemophilia. The precursor of a proteolytic enzyme capable of dissolving the fibrin clot is also found in normal plasma and it may be converted to the active enzyme by various agencies.

#### 5. *Nutritive Functions of the Plasma Proteins*

Blood plasma, when isolated and preserved under suitable conditions, is a relatively stable system compared to most biological tissues. In the living organism, however, it is subject to perpetual interchange with other substances as it flows through the tissues and comes into intimate contact with them. In a man, even at rest, the entire volume of the blood is circulated from the heart through the tissues and back to the heart in a period of the order of one minute. In a man undertaking strenuous activity, the rate of blood flow may readily rise to three or four times this amount so that an average element of blood may complete the circuit of the system in a matter of fifteen to thirty seconds. In order to achieve such a total rate of flow, given the relatively slow rate of progress of the minute amount of blood that passes through an open capillary in the course of one minute, a vast number of capillaries is required; their total number in a man may be of the order of magnitude of  $10^{11}$ . The total area of the capillaries in the muscles of an average man has been estimated by Krogh (120) as approximately two acres, and interchanges between blood and tissues are perpetually proceeding across this vast area. Thus the plasma proteins are in a steady state of dynamic interchange with the amino acids and proteins of the tissues, as revealed on the one hand by the studies of Whipple and his coworkers (139) and on

the other hand by the studies of Schoenheimer, Rittenberg and their coworkers (190, 196) who have measured the rate of incorporation of deuterium and  $N^{15}$  into the plasma proteins.

Observations by the latter method indicate that the mean half life of a plasma protein molecule may be of the order of magnitude of two weeks. Antibody proteins appear to have a half life similar to that of other proteins (95, 191). Further and more detailed studies will probably reveal differences in rates of transformation between the different protein components of blood plasma, which are not yet clearly apparent from the earlier work. However, it is abundantly clear that all of these protein components are undergoing change at a relatively rapid rate. The detailed mechanism of the transformations involved is still for the most part obscure, but there is a constant dynamic interchange of all the constituents of plasma between the blood and the tissues. Thus the injection of plasma proteins can be made to serve not only the functions of maintenance of fluid volume or the addition of antibodies for protection, but also may serve to supply the recipient with important nutritive materials when other methods of feeding are inadequate or impractical.

#### 6. *The Transport of Lipids and Other Substances in Close Association with the Plasma Proteins*

Blood plasma contains significant quantities of cholesterol, bile pigments, phospholipids, fatty acids, and various fat-soluble vitamins and hormones. These are, for the most part, not free, but are transported in the plasma in close association with certain globulin components of plasma. At least two such components are known—one representing a fraction of the  $\beta$ -, the other of the  $\alpha$ -globulins of plasma. They serve to hold in plasma, in stable aqueous solution, relatively high concentrations of materials which are virtually insoluble in pure water or aqueous salt solutions. The implications of these facts, for exchange and transport of fat-soluble materials, are obvious. The present status of our chemical knowledge of these lipoproteins is further considered in Part VI.

Serum albumin, in addition to its important osmotic function, shows specific powers of reversible combination with a large variety of molecules. These include the anions and cations of many acid and basic dyes; the anions of fatty acids and alkyl sulfonic acids, of many aromatic carboxylic acids and acetylated amino acids; sulfonamide derivatives; many types of naphthoquinone derivatives; and a variety of other compounds. All these substances show a very strong preferential tendency to combine with albumin, rather than with the other proteins of

plasma; they do not combine with albumin in all proportions, but a definite saturation capacity appears to be attained, above a certain mole ratio of added substance to albumin. Above this saturation capacity, which may differ considerably from one substance to another, the excess of added material may be bound by one of the globulin components, or may be free in the plasma. The association constants between albumin and these various substances also vary widely, depending both on the nature of the ionic groups and of the non-polar residues in the added substance. This transporting function of the serum albumin for many substances has far-reaching biological applications, as was first emphasized by Bennhold (12, 13). Moreover, many of these added substances, such as the anions of fatty acids and acetylated amino acids, greatly enhance the stability of albumin solutions, and their resistance to denaturation by heat and by reagents such as urea. All these facts are considered in further detail in Part VII.

Other specific transport functions of certain plasma proteins have already been recognized. Thus the capacity of plasma to transport iron depends on a specific globulin fraction which can be separated and highly concentrated. There can be little doubt that further intensive study will reveal other plasma fractions, as yet unrecognized, with important and specific functions.

### *7. Purposes and Advantages of Fractionation*

Elaborate and varied processes are required to separate the many components of plasma. Although a rational approach to the principles of fractionation is now possible (see Part III), plasma is such a complex system that the steps of any successful process must be worked out largely by repeated trial, with gradual and systematic variation of the conditions of fractionation. Nevertheless, the motives for persistence in the undertaking are decisive. They fall into two classes: (1) The superiority of purified plasma fractions to whole plasma for scientific study. (2) The superiority of the fractions to whole plasma for clinical and other uses (see Cohn, 35, 36, 37, 38).

Concerning the first of these factors, little need be said. It is one of the great goals of biochemistry in general to analyze systems into their components, under conditions in which the components can be separated with a minimum of damage, to learn by all possible means the chemical nature and biological function of the purified components, and eventually to show that the original system can be recreated, in all its essentials, by the proper combination of the components. For blood plasma the last step—the recreation of the total system—is a far less formidable task



than for most biological tissues. The main problems arise from the vast number of components, the close chemical similarity of many of them, and the necessity of separating each as nearly pure, and as nearly in its native state, as possible.

On the practical side, several considerations deserve attention. For many clinical purposes, especially when anemia is present, the administration of whole blood, or separated red cells in a suitable suspension medium, can meet the patient's needs more effectively than plasma or any plasma fraction. Where red cells are not needed, however, the use of plasma fractions, rather than whole plasma, often has great advantages.

(a) For each specific clinical condition, it is usually not the whole plasma, but one or more of its components, which is essential; thus the  $\gamma$ -globulin fraction contains many antibodies, some of which are of great value in passive immunization in certain infections, or in treatment; but the  $\gamma$ -globulins are relatively ineffective in combating shock, owing to their high molecular weight and low osmotic activity. Conversely, concentrated serum albumin is highly effective against shock, but completely ineffective for immunization. Economy is achieved by separating the fractions, and making each available for its specific use.

Moreover, a specific plasma fraction has a far higher activity, with respect to its specific function, than the whole plasma from which it was derived. Thus the size of the dose to be administered is greatly decreased; and this difference in size may actually be decisive in making possible the clinical use of a plasma fraction, for a purpose for which whole plasma could not even be considered.

(b) Whole plasma contains proteolytic enzymes which slowly digest other protein components of plasma. Most of the separated and purified fractions, such as albumin and  $\gamma$ -globulin, are free of these enzymes and are very stable in aqueous solution for periods of several years. Other fractions, which contain the enzymes, can be preserved dry, and re-dissolved when they are wanted for their own specific uses.

(c) The conditions for preservation in a state of maximum stability, with a minimum of denaturation, differ widely for the different components. Thus albumin is greatly stabilized in the presence of dilute sodium caprylate or of the sodium salt of acetyl-tryptophan; its solutions can be heated for as long as 10 hours at 60°C. or higher, without damage to the albumin and with destruction of any viruses which might otherwise contaminate the solution (186). On the other hand,  $\gamma$ -globulin is not stabilized at all by the substances which stabilize albumin, but it is stabilized by glycine and by certain simple sugars, which are useless for stabilization of albumin solutions.

Likewise, most of the plasma fractions can be dried from the frozen state without damage, and will redissolve to give clear and active solutions. The lipoprotein components, however, cannot be dried without breaking the intimate association between the protein and the lipid component; fatty solutions result, and the protein component becomes partly insoluble in water or salt solutions. Fractionation permits the separation of these lipoproteins without drying, so that they remain in clear aqueous solution, while other components may be prepared in dried and undenatured form to insure maximum stability.

(d) Some plasma fractions may be converted to special products, with new functions. Thus the fibrinogen fraction of plasma may be converted into fibrin foam (14) for use in hemostasis (5), fibrin film (68, 69, 70) for use as a dural substitute and for other purposes (6), and fibrinogen plastics (68). The plasma fraction containing prothrombin may be converted to thrombin, for use as a hemostatic and as a reagent for preparing fibrin foam and film. Other modified products, with specific uses, may well be developed in future from plasma fractions.

Nothing said here is intended to imply a lack of value in the use of whole plasma, which will undoubtedly remain indispensable for many purposes; but the development of plasma fractionation products has opened up a range of actual and possible applications, the full extent of which is only beginning to be explored.

## II. CRITERIA OF SEPARATION AND PURITY

### 1. *General Considerations*

The process of fractionation is, of course, impossible without adequate criteria for recognition of the separated components. These criteria may be of the most varied sorts. In earlier studies, the components were often defined simply in terms of the method employed in separating them. Thus, when ammonium sulfate fractionation was used, the albumin fraction was defined as that fraction which was soluble in half saturated ammonium sulfate. For the more accurate studies, the fractions employed were generally reprecipitated several times. However, there was seldom any direct independent evidence concerning the homogeneity of the fractions so obtained.

The components of plasma are numerous. When one considers the vast number of different antibodies, enzymes, and hormones which may be present in plasma as trace components, the number of plasma proteins capable of recognition by distinguishable tests runs into the hundreds, possibly into the thousands. Thus, the presence of more than twenty different antibodies has already been identified by systematic tests on

$\gamma$ -globulin separated from pooled plasmas derived from a mixed population of normal blood donors (Enders, 62). The number of antibodies that could be detected is certainly far greater than this.

The enzymes present in plasma include an acid and an alkaline phosphatase (9); serum esterase (147, 148); certain peptidases; a proteolytic enzyme, plasmin, and its precursor, plasminogen (31, 33, 111); and many others which must be present in smaller amount. Among the enzymes, also, we must certainly list thrombin and its precursor, prothrombin (146, 193): since the enzymatic nature of the action of thrombin on fibrinogen in the production of the fibrin clot appears now to be practically beyond dispute, although the nature of the reaction catalyzed by thrombin is still far from clear.

Hormones present in plasma must presumably include every hormone produced by any gland, since it is in the very nature of a hormone that it must be transmitted by the blood stream from its point of origin to the region where its specific action is exerted. It is, of course, true that the concentration of most hormones is so low that they cannot be detected in whole plasma. However, they may become readily detectable in many cases, when suitably concentrated in the process of fractionation.

Evidently it would be useless here to recapitulate all the specific tests that have been employed for the various antibodies, enzymes, and hormones found in plasma. Such tests, however, are indispensable for the study of a vast number of plasma components which are of fundamental importance, both in scientific investigation and in the clinic, although they may be present in very small amount.

We may now turn, however, to a discussion of certain methods of broader and less specific applicability: namely, electrophoretic and ultracentrifugal analyses, determinations of amino acid and other chemical groupings, and solubility measurements.

## 2. *Electrophoretic Measurements*

The fundamental principles of electrophoretic analyses by the moving boundary method of Tiselius are already so well known (215, 128, 127, 125) that no attempt will be made to explain them here. Certain considerations, however, deserve stress. An electrophoretic pattern, as photographed either by the schlieren scanning technique of Longsworth (125) or by the cylindrical lens system of Philpot (168) and Svensson (210), reveals a certain number of peaks more or less sharply resolved. The computation of the mobilities of the different components from the position and form of the peaks, and of the relative concentrations of the components from the areas under the peaks, has been fully described

elsewhere. The possibility of observing these peaks depends upon the existence of refractive index gradients in the solution; the steeper the curve at any point, the steeper the gradient. Thus the measured areas

represent integrals of the form  $\int \frac{dn}{dx} dx$  where  $n$  is the refractive index

of the solution and  $x$  is a space coordinate in the direction of motion of the boundary. The integral as evaluated gives the refractive index increment produced by the protein component which changes its concentration at the boundary in question. From this increment, the weight concentration of protein can be inferred only if the specific refractive increment of the particular component at the boundary is known. Fortunately for such calculations, the specific refractive increments of most proteins, expressed as increase of refractive index of the solution per gram of added protein per 100 ml. of solution, are very nearly the same, being approximately 0.00185 for light of wave length near 5890 Å (Sodium D-line). Even proteins containing a very large amount of bound lipid, such as certain  $\alpha$ - and  $\beta$ -globulins of plasma, have specific refractive increments not very different from those of proteins made up completely of amino acid residues.\* All data for the relative concentration of protein components in plasma or its fractions, derived from such optical measurements, must be interpreted in the light of these facts. Furthermore, it is necessary, when electrophoretic schlieren diagrams are obtained with two or more very closely overlapping boundaries, to adopt some rather arbitrary conventions in assigning a given fraction of the total area to each of the recognizably distinct components. Not infrequently, in certain plasma fractions, the assignment becomes so arbitrary, and the proteins are so poorly resolved, that no definite conclusions can or should be drawn as to the relative concentrations of the different components, or even as to the number of distinguishable components present.

Apart from these technical difficulties, recent investigations, particularly those of Svensson (209, 210) and Dole (54), have revealed certain fundamental principles concerning moving boundary measurements, which had not been formulated by earlier workers.† These authors have given a general proof for the statement "that a system that contains  $n$  ions will, in general, form  $n - 1$  boundaries, whether or not any ions

\* However, the refractive index increment per gram of protein nitrogen must obviously be much larger for such lipoproteins than for ordinary proteins of higher nitrogen content. For a critical study of this point, as it concerns various plasma protein fractions, see Armstrong, Budka, Gross and Hasson (3).

† Experimental verification of Dole's conclusions, for systems containing several different simple inorganic ions, has been provided by Longsworth (126).

disappear over a boundary. If the system contains  $p$  anions and  $q$  cations, there will generally be  $p - 1$  boundaries with negative velocities, and  $q - 1$  with positive velocities. A system containing  $n$  ions may, however, form less than  $n - 1$  boundaries under specific conditions" (54, p. 1124). In general, the observed mobility of a boundary does not correspond exactly to the mobility of any single individual ion in the system, but is a function of the mobilities of all of them. In certain cases, the mobility of a boundary may differ very considerably from that of any individual ion in the system. Such boundaries have been termed "false" boundaries by Svensson. The false resting ( $\delta$  and  $\epsilon$ ) boundaries have, of course, been well known for some years, but the possible presence of false *moving* boundaries has also been stressed by Svensson, who has also formulated in detail the choice of optimum conditions for the attainment of true boundaries, from which the mobilities and concentrations of individual protein ions may be correctly deduced.

No attempt will be made here to reproduce the mathematical discussions of Dole and of Svensson, but some conclusions of fundamental importance may be briefly stated.

1. Apart from the exceptional case of two ions with the same mobility, it has been established that each ion changes its concentration at each boundary.

2. If the true mobility of any protein component is to be obtained, it must be absent on one side of the boundary across which its mobility is being measured. The necessity for this condition has been generally recognized, and need not further be discussed here.

3. The boundary anomalies are least for ions of high refractive increment relative to their total net electric charge. Thus, proteins and other colloidal ions are the most suitable for electrophoresis without pronounced boundary anomalies.

4. The buffer ion of the same charge as the protein ion being studied should preferably be a slow one. "If no buffer ions slower than the leading (protein) ion are available, both cations and anions in the buffer should be chosen with low mobilities, but it is especially important that the ions of the same charge as the leading ion should be slow" (210). The usefulness of slow buffer ions was discovered experimentally by Longworth, Shedlovsky, and MacInnes (128), who introduced the diethylbarbiturate buffers which have since been so widely used. Buffers of this type were useful for several other reasons, but it was also observed that the boundary anomalies were less pronounced with such buffers.

5. The following rule was formulated by Svensson: "The refractive index increment given by the leading ion is enlarged by slower surround-

ing ions of the same charge; it is diminished by faster ions of the same charge and by ions of the opposite charge" (210, p. 26). The meaning of this rule may be best illustrated by a specific example. Consider a mixture of purified serum albumin and  $\gamma$ -globulin in equal amounts. The albumin moves faster; hence on the ascending side the  $\gamma$ -globulin migrates in a medium in which it is surrounded by some of the faster albumin ions. Therefore, by Svensson's rule, the  $\gamma$ -globulin peak appears too small. On the descending side, the albumin, being faster, is moving in a medium containing the  $\gamma$ -globulin. Therefore, it follows from the same rule that the amount of the faster component appears too large. Therefore, in both limbs of the apparatus the amount of albumin appears larger than it really is, while the  $\gamma$ -globulin appears less.

6. The fundamental equations of Dole and Svensson indicate that these errors are diminished at high ionic strength and low protein concentration. If a critical examination is being made, measurements should be carried out at several different ionic strengths and several different protein concentrations, and the percentage of the total area under the peak corresponding to any component in the diagram may be plotted against the ratio of protein concentration to ionic strength. Extrapolation of the measurements to a zero value of this ratio should give the true estimate of the amount of the component present.

These general considerations have been verified experimentally by several workers. First, it was shown by Svensson (209) in a study of pig serum at 1:2 dilution that the albumin peak in the ascending limb corresponded to 59% of the total protein at ionic strength 0.10, but only to 43% on addition of 0.37 *M* sodium chloride. The high ionic strength repressed the boundary anomalies, and the latter value is to be regarded as the correct one.

Similar experiments were carried out on human plasma by Perlmann and Kaufman (167) and by Armstrong, Budka, and Morrison (4), with results of the same sort. Perlmann and Kaufman, using sodium diethylbarbiturate buffers of pH 8.6, obtained 57.8% albumin and 9.8%  $\gamma$ -globulin at ionic strength 0.1. At ionic strength 0.2, the albumin value decreased to 54.8% and the  $\gamma$ -globulin increased to 12.3. The figures at 0.3 ionic strength were almost identical with those at 0.2. The calculated values for  $\alpha_1$ -,  $\alpha_2$ - and  $\beta$ -globulin, and for the fibrinogen component, were little affected by change in ionic strength. Similarly, when the protein concentration was varied from 4.0 to 1.5 mg. N per ml., the albumin peak decreased from 58.3 to 54.6%, while the  $\gamma$ -globulin rose from 9.8 to 11.3%. The extrapolated value for albumin at high ionic strength and zero protein concentration was in the neighborhood of 53%.

TABLE I  
*Distribution of Electrophoretic Components of Human Plasma Pool No. 170 as Determined by Analysts at Several Protein Concentrations  
 in Sodium Diethylbarbiturate of pH 8.6 and Varying Ionic Strength*

$\Gamma/2$	Protein Concentration (g./100 cc.)	Albumins %	Total $\alpha$ -Globulins %	$\beta$ -Globulins %	"Fibrinogen" %	$\gamma$ -Globulins %	$\alpha_1$ -Globulins %
0.05	2.5	57.9	10.7	14.0	6.3	11.1	4.4
	2.0	57.7	12.5	14.3	6.5	9.0	3.6
	1.0	55.9	13.9	15.0	6.3	8.9	4.2
0.10	2.3	57.3	11.2	14.7	6.7	10.1	4.0
	2.0	55.1	14.2	12.3	7.8	10.6	5.0
	1.5	55.4	13.2	13.6	6.6	11.2	5.4
0.20	1.0	53.7	14.8	14.2	7.0	10.3	5.4
	2.5	53.4	12.3	15.1	6.6	12.6	6.6
	2.0	52.3	14.9	13.8	7.3	11.7	4.7
0.30	1.0	51.4	15.0	14.3	6.9	12.4	5.6
	2.0	51.3	14.4	16.3	5.8	12.2	5.7
	1.5	50.4	15.8	14.8	6.3	12.7	8.3
	1.0	51.0	15.4	14.5	6.7	12.4	6.8

From Armstrong, Budke, and Morrison (4).

It should be noted that the percentage of "fibrinogen" as given by the area under the  $\phi$  peak in electrophoresis is distinctly higher than the percentage of fibrinogen determined directly as protein clotable with thrombin. The latter value is about 4% (Edsall, Ferry and Armstrong, 59) in human plasma.

Similar observations of the effect of ionic strength on the electrophoretic distribution in bovine plasma are given by Koenig, Perrings, and Hogness (119a). However, they found the effects of varying ionic strength in phosphate buffer to be much greater than in barbiturate buffer, indicating specific interactions between protein and phosphate ions, not considered in the theories of Dole and Svensson.



TABLE II  
Distribution of Components in Electrophoretic Schlieren Diagrams of Normal Human Plasma Pools  
Analyzed in Sodium Diethylbarbiturate Buffer pH 8.6

	Albumins %	Total $\alpha$ -Globulins %	$\beta$ -Globulins %	$\gamma$ -Globulins %	"Fibrinogen" %	$\alpha$ -Globulins %
Average Values: Plasma Pools	55.2	14.0	13.4	11.0	6.5	5.3
Standard Deviation	1.3	0.8	1.6	0.7	0.6	0.5
Coefficient of Variation	2	6	12	6	9	9

From Armstrong, Budka and Morrison (4).  
See footnote to Table I.

TABLE III  
Mobilities of Electrophoretic Components of Normal Pooled Human Plasma Proteins  
in Sodium Diethylbarbiturate Buffer of pH 8.6 and Ionic Strength 0.1

Component	Albumins	$\alpha$ -Globulins	$\alpha$ -Globulins	$\beta$ -Globulins	Fibrinogen	$\gamma$ -Globulins
$U \times 10^5 \left( \frac{\text{cm}^2}{\text{volt. sec.}} \right)$	5.92	4.85	3.87	2.88	2.06	1.15
Standard Deviation	0.21	0.23	0.22	0.15	0.20	0.20

From Armstrong, Budka and Morrison (4).



The findings of Armstrong, Budka, and Morrison were essentially similar. They are reproduced in detail in Table I. They concluded that the contribution of albumin to the total refractive increment of unextracted pooled human plasma proteins, by the electrophoretic method, was  $51 \pm 1.5$  per cent. On the basis of protein nitrogen calculations, this figure yielded an estimate of  $54 \pm 1.5$  per cent albumin nitrogen as per cent of total protein nitrogen, in pooled normal human plasma.

Armstrong, Budka, and Morrison also studied known mixtures of purified plasma protein components, in known proportions. The systems studied included mixtures of albumin with  $\alpha_2$ -globulin, with one of the  $\beta$ -globulin fractions, and with  $\gamma$ -globulin. The results, for mixtures of albumin with equal amounts of fibrinogen and of  $\alpha_2$ -globulin respectively, at ionic strength 0.05 and pH 8.6, are shown in Figure 1a. The apparent electro

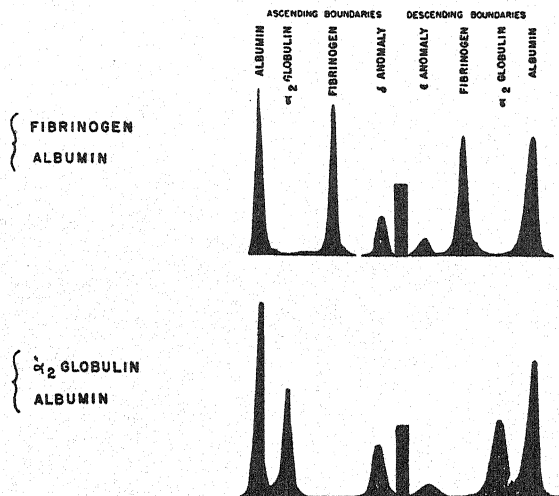


Fig. 1a Electrophoretic studies on known mixtures of purified human plasma protein components. From Armstrong, Budka and Morrison (4).

phoretic distributions, for the fibrinogen-albumin mixture, are very close to the true values as known from the amount of each component added. In the mixture of albumin and  $\alpha_2$ -globulin on the other hand, the two components have much more nearly the same mobility, and the apparent percentage of albumin, as judged from the relative size of the two peaks in the diagram, is 7 per cent greater than the true value. However, when the ionic strength was increased to 0.3, and the protein concentration reduced to 1 per cent, the values obtained from electrophoresis coincided exactly with those deduced from the amounts of the components added.

In Figure 1b, the observed deviations in these systems are plotted against the ratio of protein concentration to ionic strength. It will be

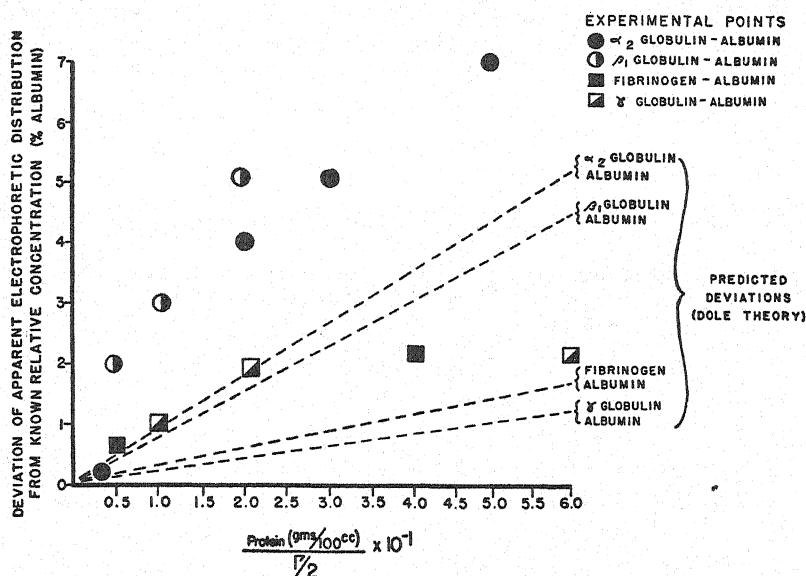


Fig. 1b. From Armstrong, Budka and Morrison (4).

seen that the observed deviations are always in the direction to be expected from Dole's theory, but their magnitude is rather greater than the theory would predict. These results suggest specific interactions between the components of the system, not yet predictable from theory.

Electrophoretic studies on human and other plasmas have seldom been carried out with the refinements and corrections involved in these studies. For most purposes, it is of major importance to obtain systematic comparisons under standard conditions, rather than absolute values. The

TABLE IV  
*Electrophoretic Distribution of Proteins in Various Animal Sera*

Normal Sera	Alb.	$\alpha$	$\beta$	$\gamma$
Cow	41.0	13.0	8.2	37.8
Guinea Pig	55.8	14.5	8.2	21.5
Horse	32.1	13.8	24.2	29.9
Pig	42.4	16.0	16.3	25.3
Rabbit	59.6	7.05	12.0	21.35
Sheep	57.3	10.5	7.2	25.0

Data from Svensson (210): the figures given are averages of many individual determinations (see text). Detailed data on swine serum and plasma are given by Koenig and Hogness (119); on beef serum and plasma by Hogness, Giffie and Koenig (104); on various animal plasmas by Deutsch and Goodloe (52) and by Moore (153).

data are now very numerous; the most extensive studies on pooled human plasma are those of Armstrong, Budka, and Morrison. The average values of a series of 20 human plasma pools obtained by them are given in Table II, and the mobilities of the components in Table III (p. 397).

While this review is primarily concerned with the proteins of human plasma, it is of some interest to give some examples of electrophoretic analyses of certain animal plasmas, as represented recently in the work of Svensson (Table IV) (p. 399).

It should be noted that while the average values reported by Svensson are given in Table IV, the full data for individual runs which he reports show wide individual variations. Thus for albumin in cow serum, he obtained values as low as 34.8 and as high as 47.1%, while for guinea pig serum, the albumin values varied between 46.8 and 64.4. However, making due allowances for these variations, the data still provide clear evidence that there are great differences in the amounts of the principal electrophoretic components in the plasma of different species. Other electrophoretic data on a number of different animal species have recently been reported by Deutsch and Goodloe (52) and by Moore (153).

A final comment should be added concerning the chemical homogeneity of the fractions separated by electrophoresis. Such a separation is based on a single criterion, that of mobility in an electric field. Proteins which differ widely in size, in shape, and in chemical composition may happen to have the same mobility. Abundant evidence is now available—some of which is presented later in this review—that most of the electrophoretic components of plasma are far from homogeneous. Among both  $\alpha$ - and  $\beta$ -globulins, for example, there are known to be lipoprotein components containing large amounts of cholesterol, phosphatides, and fatty acids, closely bound to the protein. Electrophoretically, these lipoproteins move with other  $\alpha$ - and  $\beta$ -globulins which are virtually lipid free and are completely different in their solubility behavior and probably also in their amino acid composition.

Furthermore, preparations of human  $\gamma$ -globulins, which are more than 99% pure by the criterion of electrophoresis, show distinct inhomogeneity when studied in the ultracentrifuge, containing appreciable quantities of a component sedimenting faster than the ordinary serum globulin. (See Part VI, Table XI). Serum albumin appears like a more nearly homogeneous protein than any of the globulins which can be resolved by electrophoresis, since the molecules appear to be of very similar, if not identical, size and shape. Yet the studies of Luetscher (129) showed that serum albumin preparations, which were electrophoretically homogeneous alkaline to pH 7, may show two or more components by

electrophoresis near pH 4. Moreover, even carefully purified serum albumin preparations commonly fail to satisfy the solubility test for a single pure component, and it is highly probable that the best preparations obtained still contain a mixture of different though similar molecules. Thus, electrophoresis, although an indispensable tool in the study of plasma proteins and their fractionation, should be regarded as only one among many such tools, and electrophoretic data should be constantly criticized and interpreted in the light of all the other information available.

No attempt will be made here to discuss the use of electrophoresis as a method for the preparative fractionation of proteins. Necessarily, the electrophoretic method is at present restricted to small scale work, and it is still extremely difficult to separate any component in high purity, except perhaps by separating a very narrow band from the central region midway between two adjoining boundaries. If high purity is so obtained, this purity must be paid for by a much diminished yield. Experimental separation of electrophoretic fractions has been employed, for instance, by Blix, Tiselius, and Svensson (16) to determine the distribution of lipids in various plasma fractions. Thereby they showed that the lipids were chiefly associated with  $\alpha$ - and  $\beta$ -globulins, a conclusion fully confirmed by the large scale chemical fractionations described in Part VI.\* However, they reported appreciable amounts of lipid in the  $\gamma$ -globulin and albumin fractions, while large scale chemical fractionation proved both these components to be virtually lipid free. Probably the discrepancy was due to inadequate purity of the electrophoretically separated fractions.

Future developments of electrophoretic separation methods will be awaited with interest; whether they can be developed to give large yields of products of high purity is at present unknown. The recent study of an electrophoretic convection method, by Nielsen and Kirkwood (158) is of interest in this connection.

### 3. Ultracentrifugal Measurements

*A and G Components.* The ultracentrifugal pattern of human and animal serum has been studied much more widely than that of whole plasma. Most of the observations hitherto made, therefore, have not dealt with the fibrinogen component, which will be discussed separately below. All of these sera show two main components, one with  $s_{20}$  approximately equal to 4.5 S, the other with  $s_{20}$  nearly 7 S. In addition, most

\*It should be remembered, however, that the chemical fractionations also demonstrated the presence of other important  $\alpha$ - and  $\beta$ -globulin components, which are virtually lipid-free.

plasmas, and certain globulin fractions, show significant amounts of a much more rapidly sedimenting component with an  $s_{20}$  value between 17 and 20 S. The exact value obtained for the latter component varies markedly with concentration, increasing toward a limiting value of about 20 as the concentration decreases.\*

The earliest extensive studies of serum in the ultracentrifuge were those of von Mutzenbecher (156). The interpretations which he offered of his results, however, have been largely modified and superseded by those of later workers, notably McFarlane (132) and Pedersen (166). McFarlane denoted the 4.5 S component as the A, and the 7 S component as the G component, to indicate their general relationship, but not complete identity, with albumin and globulin, respectively, as determined by other methods. It was noted by von Mutzenbecher, whose observations were confirmed and then greatly extended by McFarlane, that the A/G ratio in the ultracentrifugal pattern increases progressively with increasing concentration of the serum. Thus there is at least one component of the serum proteins which changes its sedimentation constant very readily as the concentration of total protein changes. The phenomena suggest a reversible aggregation and disaggregation of certain serum proteins. The component responsible for these phenomena was denoted by McFarlane as the X-component. It can generally be recognized as a bulge, making the albumin peak asymmetrical in the sedimentation diagram, but its exact position is very sensitive in the concentration of both proteins and salts in the solution under study. The recent work of Pedersen (166) has greatly clarified the nature of this component, at least as it exists in human serum (see below).

Quite apart from the X-component, certain purified plasma globulin fractions—notably some  $\alpha$ - and  $\beta$ -globulins—have sedimentation constants ( $s_{20}$ ) near 4.5 S, and are indistinguishable in this respect from albumin.†

*The 20 S Component.* This is more prominent in certain species of animals than in others; and in some species, at least, it increases with

\*The work up to 1940 in this field, as in all other matters pertaining to the ultracentrifuge, is well reviewed by Svedberg and Pedersen (208). It should be noted that  $s_{20}$  denotes the sedimentation velocity, in a field of unit centrifugal acceleration, at 20°C., in a medium of the same viscosity as water at 20° (approximately 0.01 poise). Moreover in the calculations it is conventionally assumed that the partial specific volume of the anhydrous protein is 0.749. If the actual partial specific volume differs markedly from this value, the calculated  $s_{20}$  value varies with the density of the solvent, as in Fig. 2 (p. 404) for the X-protein of plasma.

The properties of these fractions are discussed further in Part VI.

increase in immunity to certain antigens. The work of Kabat (110) indicated that the anti-pneumococcus antibodies from the horse, cow, and pig belonged in this category, the molecules having molecular weights of the order of 900,000, and very high dissymmetry factors, indicating an axial ratio of the order of 20:1. The anti-pneumococcus antibodies of rabbit, monkey, and man, on the other hand, were smaller molecules of the 7 S variety with molecular weights of the order of 160,000 and much less asymmetrical in shape.

This work will not be further described here, since it has already been reviewed by Treffers (216) in an earlier volume of this series. It should be noted, however, that molecules of the 20 S class are not restricted to any particular species of animal or to any particular class of antibodies. The ultracentrifugal studies of Oncley (162) have revealed rapidly sedimenting components in several fractions of human plasma, including components which may be electrophoretically either  $\beta$ - or  $\gamma$ -globulins. The exact relation of these large molecules to the ordinary smaller molecules of the 7 S group is still obscure. It is tempting to suppose an end-to-end aggregation of six or seven of the smaller molecules to form one larger one, but there is at present no evidence of anything like a reversible equilibrium between the two types in plasma. The general chemical and functional significance of these large molecules is, therefore, still a matter remaining to be explored.

*The X-Component.* Pedersen (166) has recently carried out important studies which have greatly clarified the nature of the X-component of human plasma. His elucidation of the problem began with a chance observation that the sedimentation constant of the X-component was much diminished when he employed as solvent a buffer of higher ionic strength than he had generally used. Further studies revealed that this change of sedimentation constant was not due to a specific effect of the phosphate buffer first employed, but that it could also be obtained with sodium chloride and other salt solutions by increasing the concentration of the added salt to 0.3 - 0.4 molar. Study of the sedimentation rate in a large number of different salt solutions showed a consistent correlation with only one property of all the solvents—the density. Progressive increase of salt concentration, with accompanying increase in density, produced invariably a decrease in sedimentation constant until, in solvents of a density approximately 1.04, the sedimentation constant fell to zero, and at higher densities it became negative. Thus the value of 1.04 represented the approximate density of the protein itself.

Examination of protein fractions separated in a preparative electrophoresis apparatus indicated that this component was a  $\beta_1$ -globulin.

Samples were also obtained by Pedersen in the preparative ultracentrifuge, by mixing 7 ml. of serum with 5 ml. of saturated  $\text{MgSO}_4$ ; this solution was sufficiently dense that the X-component sedimented in the opposite direction from the other serum proteins, and collected at the top of the cell. The enormous effect of the density of the solution on the sedimentation rate of this component is shown in Fig. 2.

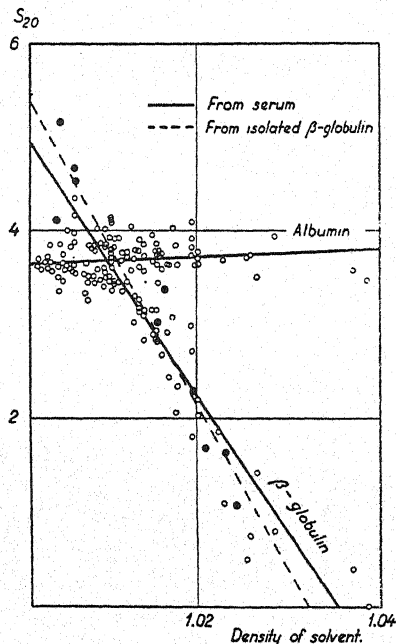


Fig. 2. The variation of  $s_{20}$ , for albumin and  $\beta$ -lipoprotein (X protein) with the density of the solvent.

Open circles denote values obtained in runs on whole serum. Solid circles denote values on runs on X-protein previously separated from whole serum by negative sedimentation in a solvent of high density. From Pedersen (166, p. 29).

The great contrast with serum albumin, for which  $s_{20}$  is virtually independent of density, shows strikingly the unusual nature of this component. Its density value near 1.04, as compared with the usual value of approximately 1.33 for a dried protein, indicates the presence of a very large amount of lipid in the molecule. From the variation of the amount of the X-component, with variation in total protein concentration, Pedersen concluded that "the  $\beta$ -globulin ('X-protein') is a reversibly dissociable component formed by albumin, globulin and some lipids with a total particle weight of the order of magnitude  $10^6$ " (166, p. 32). Since this component has now been obtained in large quantity and high

purity by the low salt, ethanol, low temperature fractionation methods described in Part VI, a further discussion of its chemical nature is given there.

It is an extraordinary fact that this  $\beta_1$ -lipoprotein has hitherto been found in high concentration only in human serum. No similar component has been separated from any of the animal plasmas yet obtained, and the fractionation methods, which have yielded large amounts of X-protein from human plasma, have given extremely little, if any, corresponding lipoprotein from the animal plasmas hitherto investigated. Thus the nature of the reaction which causes a progressive change, with serum protein concentration, of the A/G ratio, in sera other than human, still remains to be elucidated. The existence of these phenomena is abundantly clear from the work of McFarlane (132). Pedersen concludes: "It must be supposed that part of the globulin, in these sera also, combines with the albumin. It seems likely, however, that the densities of these compounds are such that they will be hidden in the albumin peak" (166, p. 33).

#### *Fetuin*

The work of Pedersen (165, 166) revealed another component of unusual interest in the fetal sera of calves, sheep, and horses, but present only in traces in human fetal sera. This component, designated as fetuin by Pedersen, has a low  $s_{20}$  value, near 3.2, a molecular weight of approxi-

mately 50,000, and an  $\frac{f}{f_0}$  value of 1.6 - 1.8, indicating a decidedly asymmetrical molecule. In spite of very extensive studies, he did not succeed in preparing it completely free of other serum components, and much still remains to be learned about its chemical properties. Its isoelectric point is in the acid range, near pH 3.5 (Pedersen, 166a); it is thus the most acidic of all the plasma proteins hitherto identified. The presence of fetuin can still be readily detected in the sera of calves and other young animals, but its amount diminishes rapidly with age, and it can no longer be detected in the adult animal.

#### *Fibrinogen*

This component, present in plasma but not in serum, is of fundamental importance, but as yet the data available concerning it are few. Holmberg (105) has reported for human fibrinogen, prepared by repeated salting out with ammonium sulfate, sodium chloride solution and phosphate buffer,  $s_{20} = 8.5$  S,  $D_{20} = 1.1 \times 10^{-7}$ , and  $M \approx 700,000$ . The fibrinogen preparations obtained in this laboratory by the low salt, low temperature ethanol precipitation methods discussed in Part VI, have been studied extensively by Oncley (162). He has found a considerable



degree of heterogeneity, even in fibrinogen preparations which were almost completely clottable with thrombin. There is a principal component with  $s_{20} = 8.5-9$  S, and two other components of 14 and 27 S, respectively. The significance of the larger components here is even more obscure than with the serum globulins. They may, of course, represent early aggregation states of the fibrinogen molecule, and thus may be small rudimentary precursors of the fibrin clot itself, but no definite information is yet available, to confirm or refute this suggestion.

Determinations of sedimentation and diffusion constants, and of molecular weights, of purified plasma protein fractions are presented in Part VI.

#### 4. *Chemical Analyses of Protein Fractions*

Direct analyses of the content of certain chemical components are often very powerful tools in the discrimination between protein fractions. It was established by the work of Hewitt (98, 99, 100) and McMeekin (137, 138) that different albumin fractions from horse serum differ widely in carbohydrate content. Both carbohydrate-containing and carbohydrate-free fractions were prepared by these workers; and a crystalline component of each class was obtained by McMeekin (see Part V). All these preparations were very similar as regards molecular size and shape. In contrast, all fractions of human serum albumin appear to be relatively carbohydrate-free; the carbohydrate present in the human plasma proteins is found principally in various globulin fractions.

The content of lipoproteins in many plasma fractions is readily followed by determining the cholesterol content, which may be extremely high (of the order of 20%) in some of the lipoproteins of plasma; these include both an  $\alpha$ - and a  $\beta$ -globulin component. Both of these, in addition, contain large amounts of phosphatides and some fatty acid, so that phosphorus and fatty acid determinations may also be of great value as analytical tools in discriminating these from other plasma protein components.

Other special types of chemical analysis are useful in particular cases. Thus it has been recognized for some time that the small amount of iron which plasma is capable of transporting is associated largely with one of the globulin components. Determination of the iron binding capacities of certain fractions, therefore, has been found useful in determining the presence and amount of this particular component (see Part VI).

However, the more uniformly applicable methods of analysis are obviously those involving determination of amino acid content. In the past, unfortunately, the number of such analyses that can readily be

applied during a fractionation process was very limited. Most of the methods available depended on isolation procedures; they were tedious, time consuming, and often inaccurate, and they required larger amounts of protein than could generally be spared for purely analytical purposes. Colorimetric determination, however, of such components as tyrosine and tryptophan have several times been employed as a control on fractionation procedures. The amounts of material needed for such colorimetric determinations were small, and the methods themselves were rapid. The accuracy of the results might be open to question, but if carried out by a standard procedure, the results from one run to another should be at least comparable. A notable example of the use of such methods was the determination of tyrosine and tryptophan in various fractions of gliadin in the extensive studies of Haugaard and Johnson (91).

Recent advances have enormously widened the range of applicability of amino acid determinations as a control on fractionation. Not only have colorimetric procedures been revised and increased in accuracy, but the development of microbiological and chromatographic methods of assay now permits the quantitative determination of almost any amino acid residue, using very small amounts of protein, of the order of 10 to 100 mg. The results are rapid and, by some at least of these methods, appear to be trustworthy. Since the general principles of all these methods have been reviewed in the previous volume of this series, by Martin and Synge (142) and by Snell (197), no further discussion of them need be given here.

In addition to these methods, titration curves permit the evaluation of total free carboxyl groups in protein preparations; they also permit the determination of the total free cationic groups (histidine, arginine, lysine and any free  $\alpha$ -ammonium groups that may be present.) The imidazole groups of histidine, the guanidinium groups of arginine, and the total free amino groups ( $\alpha + \epsilon$ ), can also be inferred separately from the titration. The methods used involve determination of the total acid-binding capacity, the effect of formaldehyde and of temperature on the titration curve, and other special devices (Cannan, 26; Cohn and Edsall, 39, Chapter 20). Important differences between the titration curves of certain serum globulin fractions were found by Green (82).

### 5. *Solubility of Protein Fractions*

The entire process of fractionation rests, of course, upon differences in the solubility of different components. Moreover, in highly purified individual proteins, the solubility test (Northrop, 159; Herriott, 97; Pirie, 171) is probably the most delicate single criterion of adequate purity.

However, as a routine tool in the analysis of protein fractions, solubility has been little used. In protein mixtures, the amount of protein dissolved increases rapidly with increase in the total quantity of protein in the system; and it is extremely sensitive to small changes in pH, ionic strength, temperature, and the concentration of many components in the solvent. Therefore, solubility, as a *criterion of fractionation*, will not be further considered here.

#### 6. Other Methods of Analysis

Other techniques might be, and occasionally have been, employed as controls on the process of protein fractionation. These methods include determinations of viscosity, diffusion rate, osmotic pressure, dielectric dispersion, and double refraction of flow. All of these methods\*, however, have been used chiefly for the characterization of purified proteins, rather than as controls on the process of fractionation. Their possible use for the latter purpose, however, should be seriously considered when special problems arise; success in fractionation often depends on the use of hitherto untried methods of approach, both in method of separation and in assay of the product. However, it may be reiterated here that the best methods of assay, when they can be used, are generally those which are highly specific for a particular component—enzymic or hormonal activity, or the capacity to bind chemical compounds of a particular class.

### III. SOME GENERAL PRINCIPLES UNDERLYING THE METHODS EMPLOYED FOR PROTEIN FRACTIONATION

The separation of more or less homogeneous protein fractions from the complex mixtures of proteins found in nature is still in many respects an art rather than a science. Certainly, the methods employed in such fractionations were largely developed, beginning with the work of Denis approximately a century ago, long before the chemical nature of proteins was even approximately understood. In spite of this, the methods actually employed in fractionation often yielded very satisfactory preparations by purely empirical techniques. Gradually, however, it has become apparent that the solubility of proteins bears a definite relation to their chemical structure, and a rational approach to many of the principles employed in protein fractionation procedures has become possible. Some of these principles deserve further consideration here†.

\*The principles of all these methods are extensively discussed in Cohn and Edsall (39). Concerning double refraction of flow, see also (58).

†A very detailed presentation of the factors determining the solubility of substances of this class is given by Cohn and Edsall (39), especially Chapters 8-12, inclusive; 23 and 24. A briefer discussion is given by Edsall in Schmidt's book (189, Chapter XVI).

### 1. *Thermodynamic Aspects of Solubility*

At equilibrium in a saturated solution, the chemical potential, or partial molal free energy, of the solute must be the same in the solution as in the solid phase\*. If we consider two different saturated solutions, therefore, both in equilibrium with the *same* solid phase, the chemical potential of the solute must be the same in both. The chemical potential ( $\mu$ ) and activity ( $a$ ) are related by the equation  $\mu - \mu_o = RT \ln a$ , where  $\mu_o$  is the chemical potential of the substance in the standard state. Hence, if the same standard state is chosen for all the solutions considered, the activity of the solute must be the same in all.

The activity of a component in solution may be considered as the product of two factors: the concentration—which here we shall generally express as mole-fraction—and the activity coefficient ( $f$ ). In an ideal solution, the vapor pressure or activity of each component is proportional to its mole-fraction ( $N$ ) in the solution and  $f$  may be taken as unity. If the component is denoted by the subscript  $i$ , then

$$(a_i)_{ideal} = N_i \quad (1)$$

if we choose the component  $i$ , in the pure liquid state at the same temperature, as the standard state of unit activity (for further details see, for instance, Lewis and Randall, 123, Hildebrand, 101). Thus, in an ideal solution, the activity of any component increases directly as its mole-fraction. If its mole fraction is small, its activity is also directly proportional to its concentration in mole/l.

In an actual, as in an ideal, solution, the activity of any component, in a solvent of fixed composition, always increases with its concentration. If the solution is sufficiently dilute, this increase is a linear function of the concentration (or mole fraction), of the solute, but it is not in general linear at higher concentrations. Moreover, if we consider the same solute in two different solvents, the ratio of activity to concentration, even in dilute solution, may be very different in the two cases. Consider, for instance, the distribution of glycine, in dilute solution, between water and benzene. If the concentration in the aqueous layer is (say) 0.1  $M$ , the concentration in the benzene layer is almost vanish-

\* If the solute is a volatile substance, this may also be expressed by the statement that its vapor pressure must be the same in both phases. It is immediately obvious that this condition is necessary if equilibrium exists. The chemical potential,  $\mu$ , is related to the vapor pressure,  $p$ , by the equation  $\mu + RT \ln p = \text{const.}$ , provided the vapor obeys the perfect gas law. Therefore, if the constant of this equation which determines the standard state is chosen as the same for both media, the chemical potentials must be the same in both, as the partial pressures are the same.

ingly small. Yet the *activity* (or chemical potential) of the glycine in both layers is the same, if equilibrium is attained\*.

If we denote the activity of the glycine in aqueous solution as  $(a_G)_W$ , and in benzene as  $(a_G)_B$ , then, in sufficiently dilute solution:

$$(a_G)_W = f_W N_W \text{ and } (a_G)_B = f_B N_B \quad (2)$$

Here the  $N$ 's denote the mole fraction of glycine in water and benzene, respectively, and the  $f$ 's denote proportionality constants, (activity coefficients) relating activity to mole fraction. One of these constants may be arbitrarily chosen; it is convenient to make the choice so that  $f_W = 1$  in a very dilute aqueous solution†. At equilibrium  $(a_G)_W = (a_G)_B$ , and hence from (2):

$$f_B = N_W / N_B \quad (3)$$

Hence the activity coefficient in the benzene solution is the distribution coefficient of the glycine between water and benzene. Actually, this coefficient is so large that it is almost impossible to measure; it is probably much greater than  $10^6$ ‡. A similar distribution coefficient for glycine between water and ethanol cannot be measured directly, since water and ethanol are miscible in all proportions. However, it can be determined from the relative solubility of glycine in the two media, and is not far from 2500.

These vast changes in the solubility (or activity coefficient) of an amino acid from one medium to another are even more characteristic of the proteins. They are similar, both in character and in order of magnitude, to the changes observed in the same media in the relative solubility of inorganic salts. In both cases, the fundamental explanation is the same: salts, amino acids and proteins all contain electrically charged ionic groups for which the electrical free energy (and therefore the activity coefficient) increases enormously as the dielectric constant

\* In this discussion, we presuppose that the same standard state is chosen to define the activity in both solvents. The different standard states that may be chosen, for convenience under different circumstances, are thoroughly discussed by Lewis and Randall (123).

† This choice thereby defines the standard state for glycine, by the statement that in very dilute aqueous solution

$$(a_G)_W = N_W$$

‡ A simple electrostatic calculation, considering the electrical free energy of the glycine dipolar ion in water (dielectric constant near 80) and in benzene (dielectric constant near 2) suggests that  $f_B$  may be of the order of  $10^{30}$  (see Edsall, ref. 39, p. 108-110).

of the solvent is lowered. Inorganic salts are composed of separate ions, each carrying a net charge; isoelectric amino acids and proteins carry one or more pairs of oppositely charged ionic groups separated by a considerable distance, so that the molecule as a whole is electrically neutral. This electrical neutrality of the whole molecule produces important modifications of the quantitative relations involved, as compared with those found for separate ions; but the general qualitative features of relative solubility in different solvents are the same for salts, amino acids and proteins. The largest effects of all are observed in the proteins, because of their great size and the very large number of positive and negative groups which they carry. Thus, a relatively small change in the composition of the solvent may produce a great change in the solubility of a protein. These effects are highly specific; differing markedly from one protein to another, according to the configuration of the molecules, and the numbers and positions of the various charged groups.

From measurements on the solubility of a substance in two different media, its relative activity coefficient in the two media may be deduced by the same reasoning embodied in the derivation of equation (3). This reasoning, however, involves the important proviso that the same solid phase must be in equilibrium with each of the two solutions under consideration. This condition is, in general, not fulfilled in solubility experiments on proteins; the composition of protein crystals is known to vary somewhat with variation in the amount of salt, water and other substances in the medium in equilibrium with the crystal. Nevertheless, these variations appear often to produce little change in the activity of the protein component of such a protein crystal, since independent measurements of the activity of proteins by other methods give results in general accord with those of the solubility method (on this subject see ref. 39, p. 619-622).

In any case, however, the intermolecular forces in the solid in equilibrium with a solution have effects on solubility which are of fundamental importance and deserve special discussion.

## *2. Influence of Crystal Lattice Structure on Solubility*

It is useful to attempt to form a molecular picture of the process involved when a molecule is removed from the crystal lattice of a solid and passes into solution. Changes in free energy are necessarily involved at several steps in the process: (1) The molecule must be removed from its surroundings in the crystal lattice. The free energy of this process is the free energy of sublimation, and increases with the strength of the bonds which bind a molecule in the lattice to its neighbors; (2) In order

that the molecule may dissolve in the solvent, a hole must be created in the solvent, involving a separation of solvent molecules from one another. The free energy change in this process is determined by the same forces that determine the work of vaporization of the solvent; (3) The solute molecule is then inserted into the hole. In contrast to the first two steps, which involve the expenditure of free energy, this involves a free energy release, the magnitude of which depends upon the strength of the attractive forces between the chemical groups in the solvent and solute molecules. Qualitatively it is clear that a substance which forms a crystal of high lattice energy will tend to have low solubility in all solvents, excepting those which undergo very great chemical interactions with the solute molecules\*.

Even the amino acids are relatively complex structures from the point of view of these solvent-solute interactions, and the proteins are, of course, enormously complex. For instance, if one compares two amino acids such as glycine and leucine, it is apparent that they have a general structure in common, namely the  $\alpha$  carbon atom, with an attached  $\text{COO}^-$  group carrying a negative charge, and an attached  $\text{NH}_3^+$  group carrying a positive charge; also, each has one hydrogen on the  $\alpha$  carbon. However, leucine contains, in addition, a rather bulky alkyl group, which is absent in glycine. Both molecules, if isoelectric, have zero net charge, but possess two ionic groups of opposite charge, which produce powerful interactions with neighboring molecules; these exert a profound influence on solubility. On the one hand, they lead to intense interionic attractions in the crystal lattice, which are responsible for the high melting points of the amino acids, and for their extreme insolubility in all nonpolar solvents. On the other hand, when the amino acid molecule is dissolved in water, these groups exert intense electrostatic attractions on the surrounding water dipoles, causing them to become oriented and tightly packed around the charged groups. The net result is an enhancement of solubility in such a highly polar solvent as water.

The crystal lattice energy is thus a factor of fundamental importance in all considerations of the solubility of amino acids and proteins. Un-

\* The subject matter of this paragraph is formulated with more precision, and in much greater detail, in ref. 39, Chapter 8.

† In considering the solubility of proteins in a solution equilibrated with an amorphous solid phase, one may question whether the term "crystal lattice energy" is strictly appropriate. However, even in noncrystalline solids, strong intermolecular forces must exist between the molecules of the solid phase; and these are in essence not very different from those in a true crystal lattice with its highly regular structure. The qualitative outlines of the argument given here therefore may be considered as quite general. It is, of course, well known from X-ray and



fortunately, the magnitude of this energy in such molecules is very difficult to measure, or to predict from general considerations. We know from the presence of numerous ionic groups, both positive and negative, in these compounds, that their crystal lattice energies must be large, and all their physical properties are such as would be expected from this fact. However, it is not possible at present to deduce from other considerations the fact that glycine (for instance) is very soluble in water, while cystine is very insoluble. Obviously this difference reflects, among other things, difference in crystal lattice energy. High crystal lattice energy is a complicated function of at least two factors: (1) The geometrical pattern of the molecules which pack together to form the lattice; other things being equal, shapes which permit very close packing tend to increase crystal lattice energy. (2) The tendency of molecules, which contain large numbers of positive and negative ionic groups, to form crystals with a high lattice energy. This second factor, however, depends greatly upon the first, since it is not only the nature and the number of the groups involved, but, more particularly, the nature of the relative configurations which it is possible for neighboring molecules to assume, as they come together in the formation of the crystal, which determines the magnitude of their interaction. On considering the structure of cystine in the light of these principles, one might reasonably infer that the rather symmetrical shape of the molecule and the presence of four charged groups, two positive and two negative, should permit the formation of a very compact lattice structure, in which the adjoining cystine molecules are held together by very intense attractions between oppositely charged groups. Unfortunately, the structure of the cystine crystal is not yet known in detail, although some important X-ray diffraction measurements on it have been carried out by Bernal (15) which lead to general conclusions in harmony with the argument just presented. The only detailed and complete studies on crystal structure of amino acids and related compounds are those of Corey and his co-workers, on diketopiperazine (46), glycine (2) and alanine (122), which will be discussed in a later volume of this series. It is important to emphasize here, however, that the details of

analytical studies of protein crystals that such crystals normally contain large amounts of water and also considerable amounts of solid, if salt is present in the mother liquor in equilibrium with the crystals. In this respect, protein crystals differ from those of amino acids, and the work involved in separating protein molecules from a crystal lattice is a function of the intermolecular forces between all the different ions and molecules present in the crystal. Furthermore, the process of dissolution of the crystal involves the passage, not only of protein molecules, but also of all the other constituents of the crystal into solution; and this fact must be adequately taken account of in any complete analysis of the process.



packing in the crystal are determined largely by oriented hydrogen bond formation, linking N-H groups of one molecule to oxygen in another, in such a manner that the hydrogen in the -NH---O bond is nearly on the line connecting nitrogen to oxygen. This is true for the -CO-NH- groups in diketopiperazine, a  $\text{-C=O}$  group of one molecule being bonded to an -NH of its neighbor; it is also true for the charged  $\text{-NH}_3^+$  and  $\text{-COO}^-$  groups in glycine and alanine, although the general packing plan is very different in many ways for the two amino acids. Thus the relative *direction* in which different molecules align themselves in a crystal is largely determined by the requirements of hydrogen bond formation; but the *magnitude* of the resulting intermolecular forces is determined largely by the net charge carried by the N and O atoms.

### 3. Effects of Groups Found in Proteins on Relative Solubility in Different Solvents

The ionic groups found in proteins—the negatively charged carboxyl groups of free aspartic and glutamic acid residues, and any  $\alpha$ -carboxyl groups that may be present\*; the positively charged  $\epsilon$ -ammonium group of lysine, the guanidinium group of arginine, and the imidazolium group of histidine, and any  $\alpha$ -ammonium groups that may be present—tend to increase the strength of binding in the crystal lattice, provided they lie adjacent to other groups of opposite charge in the nearest neighboring molecule. In case they are brought close to another group of the *same* charge, however, the resulting repulsion weakens the strength of the lattice. When the protein is brought into aqueous solution, *all* the charged groups exert attractive forces on the water dipoles, the latter adjusting their orientation so as to attain a minimum of potential energy. Thus these groups, in general, enhance solubility in water; but they diminish it in solvents of low dielectric constant, in which the electrical free energy—and hence the activity coefficient—of the molecule is greatly increased. Uncharged but polar groups, such as the peptide linkage, or the hydroxyl groups of serine and threonine, have a similar but weaker effect; they slightly enhance solubility in water relative to that in such solvents as ethanol.

On the other hand, non-polar alkyl and aryl groups definitely increase solubility in organic solvents, and tend to diminish it in water. In a homologous series of amino acids, the effect of a methylene group, for instance, can be expressed by a very simple rule, namely, that "each  $\text{CH}_2$  group in side chains terminating in a methyl group may be thought

\* In strongly alkaline solution, the phenolic group of tyrosine and the sulfhydryl group of cysteine are also negatively charged.

of as increasing solubility in ethanol relative to that in water 3-fold" (Cohn and Edsall, 39, p. 205).

Thus, while glycine is about 2500 times as soluble in water as in ethanol, alanine is only about 750 times as soluble, and  $\alpha$ -amino caproic acid (with three more  $\text{CH}_2$  groups than alanine) is only about 25 times as soluble. The phenyl group, as in phenylalanine, has approximately the same effect as three methylene groups. However, important as the effects of the non-polar groups are, the effects of the ionic and polar groups in amino acids and proteins are predominant, as is shown by their very low solubility in non-polar solvents relative to water.

Not only the nature of these groups but their position in the molecule exerts a profound influence on the contribution they make to solubility. Thus, a charged ammonium group and a charged carboxyl group, if situated at a considerable distance from one another, as in  $\epsilon$ -amino-caproic acid, have a far greater influence than in the  $\alpha$ -position.  $\epsilon$ -Amino-caproic acid is about a thousand times as soluble in water as in alcohol, whereas  $\alpha$ -aminocaproic acid, as already mentioned, is only about 25 times as soluble. This shows the enormous importance of the dipole moment of the molecule in its contribution to solubility. Likewise an ionic or polar group, if situated at the end of a hydrocarbon chain or phenyl group, largely counteracts the effect which the non-polar group alone would have had on solubility.

#### 4. *Effect of Ionic Strength on Solubility*

Salts and isoelectric amino acids both commonly become more soluble in the presence of added salts. In the case of a slightly soluble salt made up of ions carrying a net charge, the effect of another salt on the logarithm of the solubility is proportional, at low salt concentrations, to the square root of the ionic strength, the effect of each ion being thus proportional to the square of its valence. If only the electrostatic effects needed to be taken into account, the theory of Debye and Hückel\* would lead to the following equation, for a salt consisting of two ions, the cations being of valence  $Z_+$  and the anions of valence  $Z_-$ .

$$\log \frac{N}{N_0} = \frac{\epsilon^2 Z_+ Z_-}{2.303 D k T} \left( \frac{\kappa}{1 + \kappa a} \right) \quad (4)$$

In this equation,  $N$  is the solubility at a given finite ionic strength and  $N_0$  is the solubility at zero ionic strength in the same solvent.  $\epsilon$  is the charge on the proton,  $D$  is the dielectric constant of the solvent,  $a$  the

\* Concerning this theory, see, for instance, Scatchard (ref. 39, Chapter 3) or MacInnes (136).

mean radius of the ions of the salt,  $k$  is Boltzmann's constant and  $T$  the absolute temperature.  $\kappa$  is defined by Equation (5)

$$\kappa = \left( \frac{8\pi N e^2}{1000 D k T} \frac{\Gamma}{2} \right)^{\frac{1}{2}} \quad (5)$$

$$\frac{\Gamma}{2} = \frac{1}{2} \sum C_i Z_i^2 \quad (6)$$

Where the  $C_i$ 's and  $Z_i$ 's denote the concentrations and the valencies respectively of all the ions in solution.  $N$  is Avogadro's number.

At very low ionic strength,  $\kappa a$  becomes negligible compared to 1, and Equation (4) reduces to the simple form

$$\log \frac{N}{N_0} = \frac{1.825 \times 10^6}{(DT)^{3/2}} Z_+ Z_- \left( \frac{\Gamma}{2} \right)^{\frac{1}{2}} \quad (7)$$

Thus the solvent effect on salt at low ionic strengths, as determined by electrostatic forces, is inversely proportional to  $D^{3/2}$ . Hence in a solvent of low dielectric constant, although the solubility of a typical salt at zero ionic strength is much smaller than in water, the increase in relative solubility when other salts are added is greater.

In the case of dipolar ions, such as isoelectric amino acids, peptides, and proteins, the effect of the salt on the logarithm of solubility is proportional to the first power of the ionic strength, instead of its square root. The general equations given by Scatchard and Kirkwood (185) and by Kirkwood (116) are very complex, depending on the size and shape of the molecule and the distribution of all the electric charges in it. For the simple case of a spherical dipolar ion of radius  $b$ , with a point dipole of moment  $\mu$  located at its center, Kirkwood (39, Chapter 12) gives for  $\log N/N_0$  as a function of the ionic strength, in mols per liter, at low ionic strength,

$$\log \frac{N}{N_0} = \frac{2\pi N e^2}{2303 D k T} \left\{ \frac{3\mu^2}{2Da k T} - \frac{b^3}{a} \alpha(\rho) \right\} \frac{\Gamma}{2} \quad (8)$$

Here the symbols have the same meaning as before, except that  $a$ , the "collision diameter," is equal to  $b$  plus the mean radius of the ions in the solution,  $\rho$  is the ratio  $b/a$ , and  $\alpha(\rho)$  is a function, tabulated by Kirkwood, equal to 1.21 when  $\rho = 0.6$ , and to 1.96 when  $\rho = 0.9$ . The first term in parentheses in Equation (8) reflects a solvent effect ("salting in"), due to electrostatic forces; the second term is a "salting out" term which increases essentially in proportion to the volume of the molecule.

It should be noted that the first or "salting in" term is proportional to the square of the electric moment of the molecule and is furthermore inversely proportional to the square of the dielectric constant of the medium. The "salting out" term varies only as the reciprocal of the first power of the dielectric constant and therefore becomes relatively unimportant, as compared to the first term, for small values of  $D$ . Kirkwood has also made calculations for an ellipsoidal model of a dipolar ion, with positive and negative charges located at the foci of the ellipsoid; in this case, the "salting in" coefficient is very nearly proportional to the first power of the dipole moment, instead of its square as in the case of a sphere. (For further discussion and references, see also Edsall, 57).

Although all these relations are derived from calculations using very much simplified models in which non-electrostatic forces are ignored, it is nevertheless true that they give a remarkably good representation of the behavior of many salts and amino acids in solutions of varying ionic strength. The solvent effect of salt on a dipolar ion is represented in Fig. 3, showing the solubility of cystine in various aqueous salt solutions.

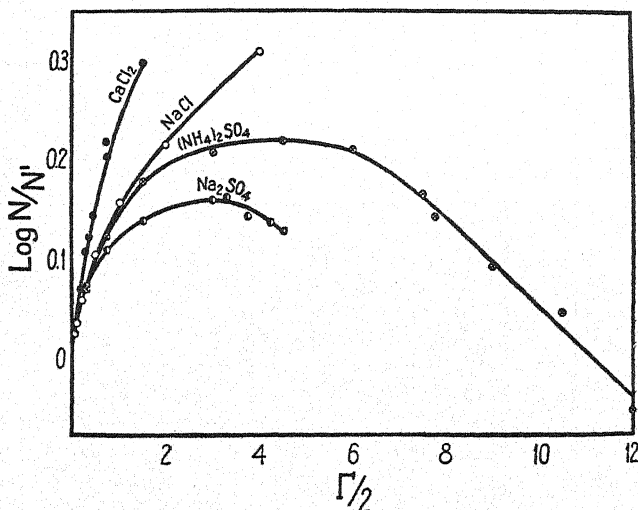


Fig. 3. Solubility of cystine in salt solution of varying ionic strength. From data of T. L. McMeekin (from Cohn and Edsall, 39, p. 243).

It will be noted that the solvent effect of the different salts shown is in all cases proportional to the ionic strength in dilute solution, but that it increases in the order, Na<sub>2</sub>SO<sub>4</sub>, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, NaCl, CaCl<sub>2</sub> (Cohn and Edsall, 39, p. 243). The effect of salts on a protein, hemoglobin, is illustrated in Fig. 4, and it will be seen that the order in which the different

salts increase the solubility is the same as for cystine. However, the magnitude of the effect is much greater for hemoglobin\*. The behavior illustrated in these figures is typical; practically every feature of the distinctive solubility of proteins is to be found also in miniature in the study of amino acids. Actually, the knowledge of these phenomena was attained far earlier in the case of the proteins, because they show these effects to a vastly greater extent, so that even the casual observer can scarcely fail to notice them.

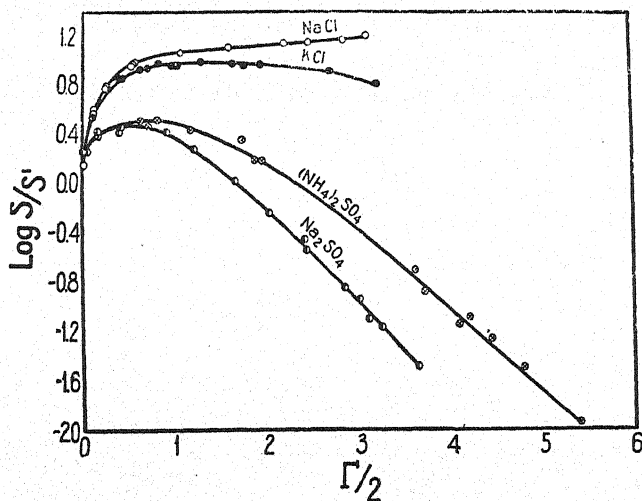


Fig. 4. Solubility of horse carboxyhemoglobin in salt solutions of varying ionic strengths. Data of A. A. Green. Note the difference in scale of both abscissa and ordinate, as compared with Fig. 3 (from Cohn and Edsall, 39, p. 608).

This increase of solubility with increasing ionic strength has been recognized since the work of Dénis as characteristic of the globulins. In many globulins, for instance edestin and myosin, the change of solubility with changing ionic strength is of a much greater magnitude than for hemoglobin, and its use in protein separation is obvious. Less widely known is the fact that even the albumins, characterized by their high solubility in water at the isoelectric point in the absence of salt, behave like globulins under conditions in which their solubility is greatly decreased. This is most readily achieved by adding a substance such as alcohol or acetone, to the water used as solvent. The decrease in the dielectric constant of the solvent produced by such an addition leads to a great decrease in the solubility of the protein in the absence of salt. It also leads readily to denaturation of the protein unless the reaction

\* Note the difference in the scale of the ordinates in Figs. 3 and 4.

is carried out at low temperature, preferably well below  $0^{\circ}\text{C}$ . However, if salt is now added to such a solution in which the solubility of the albumin is much reduced, the solvent effect of salt is very marked (71, 72). Indeed, the decrease in dielectric constant of the solvent, which is responsible for reducing the solubility in the absence of salt, also enhances the electrostatic interactions between the ions of the salt and the charged groups of the protein molecules, and thereby causes the "salting in" effect to be much larger than in water. These effects can be readily and simply illustrated in the case of glycine (39, Chapter 11), for which the solvent effect of sodium chloride in different alcohol water mixtures is plotted in Fig. 5. All the points in any given alcohol-water mixture

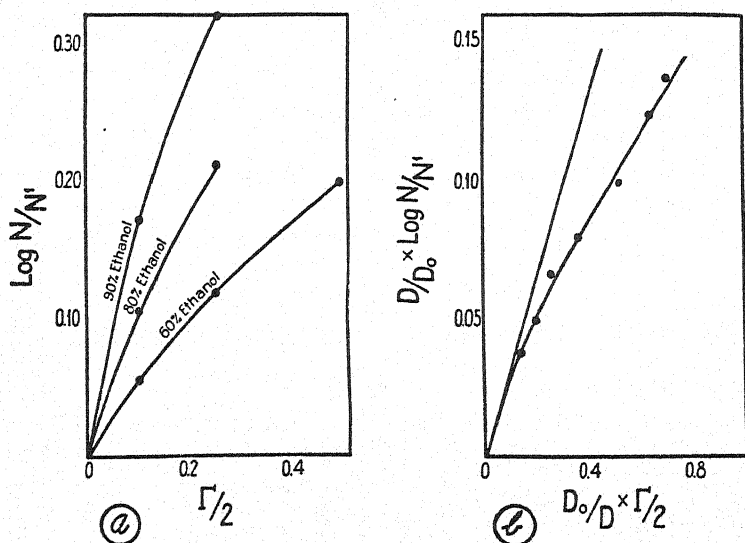


Fig. 5. Fig. 5a shows the increasing solvent action of lithium chloride on glycine with decrease in the dielectric constant of the solvent. All the data in Fig. 5a fall on a single curve when plotted as in Fig. 5b. (Data of T. L. McMeekin; see also Ref. 189, p. 918.)

are so adjusted as to start from the same origin at zero ionic strength. Actually, the solubility in the absence of salt falls very rapidly with decreasing dielectric constant, and it will be seen from the figure how the solvent effect due to added salt increases with decreasing dielectric constant. Indeed, the limiting slopes of all the different curves in Fig. 3 are directly proportional to the inverse square of the dielectric constant, as would be expected from Equation (8).

The effect of dipole moment may be illustrated by a series of solubility curves for dipolar ions of different electric moments in 80% ethanol con-

taining sodium chloride. These are plotted in Fig. 6 and their slopes reveal a regular correlation with the dipole moment of the solute as

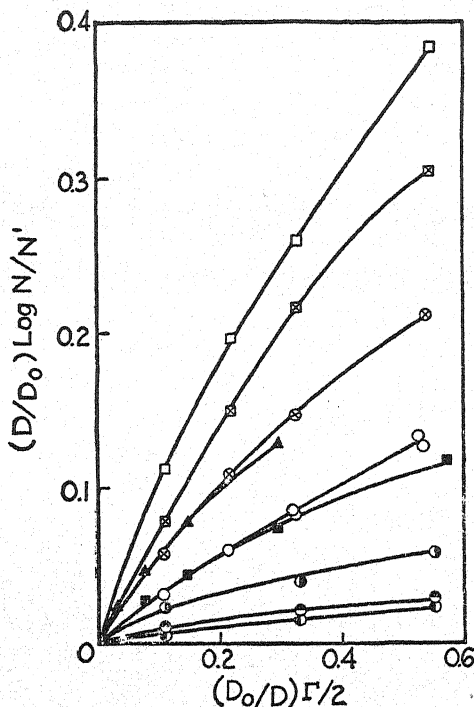


Fig. 6. Relative solubility of amino acids and peptides in 80 per cent ethanol containing sodium chloride. Reading from top to bottom, the curves are for: lysylglutamic acid; triglycine; diglycine;  $\epsilon$ -aminocaproic acid (black triangles); glycine;  $\alpha$ -aminocaproic acid (black squares); hydantoic acid of diglycine; hydantoic acid; hydantoic acid of  $\alpha$ -aminocaproic acid. The measurements on  $\alpha$ - and  $\epsilon$ -aminocaproic acids were carried out in 95 per cent ethanol containing lithium chloride.

determined from dielectric constant measurements. The correlation is illustrated in Table V, p. 421 (see Cohn, ref. 39, Chapter 11).

##### 5. Effect of One Dipolar Ion on the Solubility of Another

Not only salts, but other dipolar ions, can increase the solubility of amino acids and proteins. The solubility of cystine, for example, is increased by the addition of glycine to the solvent; glycylglycine, with its higher dipole moment, has a markedly greater effect than glycine. On the other hand, the solvent action of a series of  $\alpha$ -amino acids decreases in the order: glycine, alanine, aminobutyric acid, valine. Thus increase in the size of the non-polar side chain has the opposite effect from increase of dipole moment (Cohn, McMeekin, Ferry and Blanchard, 42; and ref. 39, Chapter 10).

TABLE V

*Salting In Constants,  $K_R$ , and Dipole Moments  
of Certain Amino Acids and Peptides*

Substance	Salting-in Constant $K_R$	Dipole Moment, $\mu$	$\mu/K_R$
Glycine	0.33	15.5	47
$\alpha$ -Aminocaproic Acid	0.33	(15.5)	(47)
Diglycine	0.58	27.6	48
$\epsilon$ -Aminocaproic Acid	0.7	29	41
Triglycine	0.8	35	44
Lysylglutamic Acid	1.2	61	51

See Cohn (39, p. 275). The dipole moments given here differ slightly from those given by Cohn, being calculated by the equation of Kirkwood (ref. 39, Chapter 12, equation 63).

Similar effects are found in solutions of some, but not all, proteins. Thus horse hemoglobin (Richards, 177) and  $\beta$ -lactoglobulin (E. J. Cohn, J. D. Ferry, and M. H. Blanchard, unpublished studies, quoted in ref. 39, Chapter 24; Grönwall, 85) become decidedly more soluble in the presence of glycine. The relative solvent effect of glycine on these two proteins, and on cystine, asparagine and glycine is shown in Fig. 7;

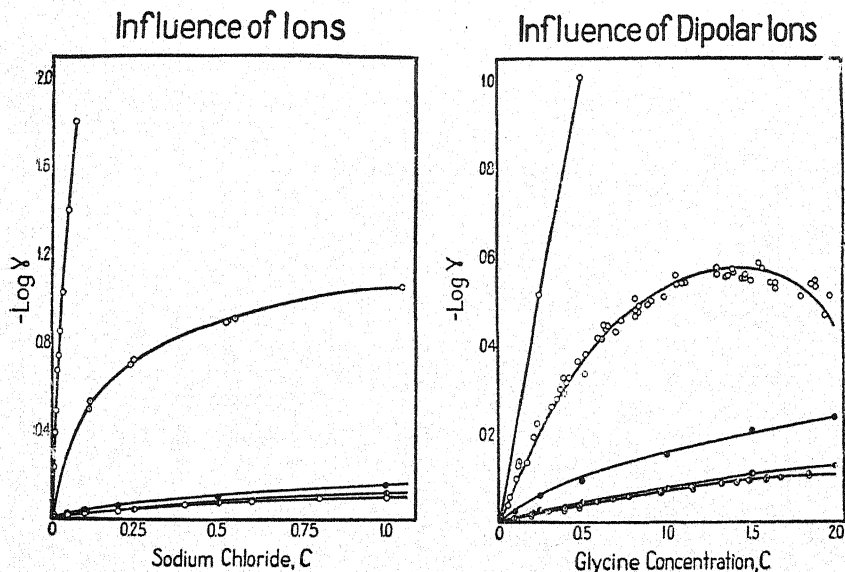


Fig. 7. Relative effects of sodium chloride (Fig. 7a) and glycine (Fig. 7b) on the solubility of certain proteins and amino acids. Note differences in scale of both abscissa and ordinate in Figs. 7a and 7b. In each figure, the steepest curve is for  $\beta$ -lactoglobulin, then below in descending order: horse carboxyhemoglobin, cystine, asparagine, glycine.



comparative data for the effect of sodium chloride on these same substances are shown in Fig. 7a. Mole for mole, the effect of sodium chloride is always greater than that of glycine; but the relative order of the curves for the different substances is the same in both figures. Grönwall (85) has also studied the effects of glycylglycine and triglycine on the solubility of  $\beta$ -lactoglobulin; the solvent effect per mole increases with increasing dipole moment of the added dipolar ion, in such a way that the solubility is approximately the same for a solution of the same dielectric constant, whether the increase in dielectric constant is due to glycine, glycylglycine or triglycine. On the other hand, Grönwall found that the solubility of serum euglobulin was not increased by any of these dipolar ions; unpublished studies in our own laboratories have led to the same conclusion for serum globulin, edestin and myosin. The meaning of this difference in behavior of different globulins is still obscure; at present it must be accepted as an empirical fact which is important in considering possible methods of protein separation.

#### 6. *The Salting Out Effect*

Neutral salts, when added to almost any solution of a non-electrolyte in water, affect the solubility of the solute. If the latter has a dielectric constant lower than that of water, the effect of the salt addition is to increase the activity (vapor pressure) of the solute, and thus to decrease its solubility. The most familiar instance to most chemists is the separation of an alcohol-water mixture into two phases on the addition of a salt such as sodium carbonate. In most such systems the solubility of the alcohol can be described by an equation of the form

$$\log \frac{S}{S_0} = -K_s \Gamma/2, \quad (9)$$

where  $\Gamma/2$  is the ionic strength of the added salt, and  $K_s$  is much smaller for calcium salts than for the corresponding sodium or potassium salts. It is much larger for sulfate or phosphate ions than for chloride, and larger for chloride than for thiocyanate. The relative salting out effect of different salts is given by the classical Hofmeister series, and is very nearly the same, no matter what the nature of the substance being salted out. For theoretical treatments of the salting out effect, which explain many but by no means all of the facts, see Debye (49, 48) and Scatchard (181, 182).

For a homologous series of organic solutes, such as alcohols, ketones, or fatty acids, the value of  $K_s$  (for a given salt) increases with the length of the hydrocarbon chain. This is correlated with the fact that the

decrease in the dielectric constant of an aqueous solution, per mole of added solute in unit volume, becomes progressively greater with increasing length of the hydrocarbon chain. However, the observed salting out effects, for molecules with large hydrocarbon groups, are generally greater than can be accounted for simply on the basis of the dielectric constant effect.

For most proteins in dilute salt solutions, the salting out effect is far more than counterbalanced by the attractive interionic forces which lead to salting in. In more concentrated salt solutions, however, the solubility of the protein passes through a maximum with increasing ionic strength, and at still higher ionic strengths there is a rapid decrease in the solubility. Over a considerable range this salting out effect can be described by the same type of simple linear equation that is applicable to similar molecules in more dilute salt solutions.

$$\log S = \beta - K_s \Gamma / 2. \quad (10)$$

Here the term  $\beta$  represents an extrapolated hypothetical solubility at zero ionic strength. It does not, of course, correspond to any measured solubility; but variation in  $\beta$ , for instance with variation of pH or temperature, furnishes a convenient index for the change in solubility of the protein with change in other variables, when the ionic strength is held constant. The order of magnitude of  $K_s$ , however, is very much greater for proteins than for simpler molecules. Examples of some of the values represented in the literature are given in Table VI. It will be seen that  $K_s$  is 10 to 20 times greater for a protein than for an amino acid. Since the value of  $K_s$  determines the change in the *logarithm* of

TABLE VI

*Values of the Salting-Out Constants,  $K'_s$ , for Amino Acids and Proteins*

Substance	NaCl	MgSO <sub>4</sub>	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	Na <sub>2</sub> SO <sub>4</sub>	Phosphate
Cystine	...	...	0.05	...	...
$\alpha$ -Aminobutyric acid	0.04	...	...	...	...
Leucine	0.09	...	...	...	...
Tyrosine	0.31	...	...	...	...
$\beta$ -Lactoglobulin	...	...	...	0.63	...
Hemoglobin (horse)	...	0.33	0.71	0.76	1.00
Hemoglobin (man)	...	...	...	...	2.00
Myoglobin	...	...	0.94	...	...
Egg albumin	...	...	1.22	...	...
Fibrinogen	1.07	...	1.46	...	2.16

the solubility, this represents an enormous change in the absolute values of  $S$  with change in ionic strength. A series of studies, notably those of A. A. Green (80) on hemoglobin, have shown that  $K_s$ , for a given protein, is virtually independent of pH and temperature. On the other hand,  $\beta$  varies markedly with pH and the rate of its variation is correlated with the steepness of the titration curve of the protein, in the pH range under consideration.  $\beta$  is generally a minimum at or near the isoelectric point of the protein, and increases on either side of the isoelectric point. Sometimes  $\beta$  passes through two or more minima; in the case of horse hemoglobin, for instance, in concentrated ammonium sulfate solution, there is a minimum at pH 6.6 and another at pH 5.4. (Sørensen and Sørensen, 202). Thus, two different proteins which may have similar solubilities at one pH value may be readily separable by salting out at another pH, provided the variation of  $\beta$  with pH is sufficiently different for the two.

#### *7. Factors Governing Variation of Protein Solubility with pH*

The effects described in the preceding paragraph can readily be generalized further. The number of charged groups in the protein molecule varies, of course, with pH. At strongly acid pH values, the histidine, arginine, lysine and  $\alpha$ -ammonium residues are all positively charged, while the carboxyl, sulfhydryl and phenolic hydroxyl groups are uncharged. In strongly alkaline solution, the converse relation holds; the protein carries its maximum net negative charge, while the groups which are positively charged in acid solution have given up protons and become uncharged. At pH values between 4 and 8 or 9, at which most fractionation processes are carried out, the net charge on a protein molecule is much smaller than in the extremely acid or alkaline range, but the total number of positive and negative groups reaches a maximum somewhere in this range. Commonly, the solubility reaches a minimum at or near the isoionic point of the protein and increases with change of pH to either side of this point.

The observed solubility of the protein represents the sum total of the concentrations of a great number of microscopically different forms—cations, anions, and dipolar ions—which are all present in a mobile equilibrium in any solution. As pH and ionic strength are altered, the relative concentrations of these different forms must also be altered. If we suppose the ionic strength to be held constant, the concentration of any one of these forms can be described in terms of the concentration of the isoelectric protein, which is directly in equilibrium with the solid phase, and of the various dissociation constants which relate the pH to the relative concentrations of the protein anions and cations. The

solubility of the isoelectric molecule, at constant ionic strength, should be very nearly independent of pH, but the concentrations of the various cations must increase as the pH is shifted to the acid side of the isoelectric point and those of the various anions as the pH is shifted to the alkaline side\*.

In Fig. 8, the logarithm of the solubility of  $\beta$ -lactoglobulin is

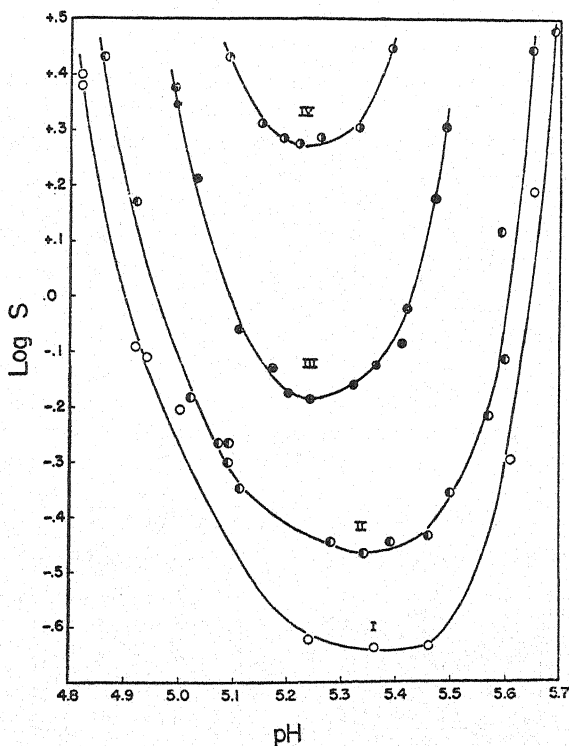


Fig. 8. Logarithm of solubility of  $\beta$ -lactoglobulin as a function of pH, at four different ionic strengths. Curve I,  $\Gamma/2=0.001$ ; curve II,  $\Gamma/2=0.005$ ; curve III,  $\Gamma/2=0.01$ ; curve IV,  $\Gamma/2=0.02$ . (Data of Grönwall, 85).

represented as a function of pH, at several different ionic strengths (Grönwall, 85). It is to be noted that, as the ionic strength increases, the minimum solubility increases rapidly. Near their minima the curves rise somewhat steeply on either side of the minimum as the ionic strength increases. This behavior can be correlated with the titration curve, which

\* Detailed studies of the effect of pH on solubility of amino acids have been carried out by Hitchcock (103) for tyrosine and by Sano (180) for cystine. The simple relations given by them may readily be generalized so as to apply to a complex molecule such as a protein with a very large number of ionizing groups.

also becomes steeper in the neighborhood of the isoelectric point as the ionic strength rises (Cannan, 26). If the logarithm of the solubility is plotted against pH at constant ionic strength, the slope of the curve at any point should be proportional to the net charge on the protein at that pH. A formulation of the relations involved has been given by Linderstrøm-Lang (see Grönwall, 85).

Except for the immediate neighborhood of the minima, the curves rise much more steeply at the low ionic strengths than at the higher; this fact does not appear easy to explain in terms of the theory, but the phenomenon is generally characteristic of the solubility of globulins.

It may also be seen in Fig. 8 that the pH of minimum solubility shifts somewhat toward the acid side as the ionic strength increases. The same effect is shown, often in a much more pronounced fashion, by many other proteins. It was noted, for instance, by Osborne and Campbell (164) that small additions of acid to isoelectric edestin, dissolved in salt solution, caused a marked decrease in solubility. Hardy (88) also noted that the effect of salt on the solubility of serum globulin, on the acid side of its isoelectric point, is commonly to decrease solubility; and he concluded that "One feature of fundamental importance, which is never obscured . . . is the antagonism between the solvent actions of salts and acids, and the additive nature of the combined solvent action of salts and alkali." Similar observations have been made by Edsall (56, and unpublished observations) on myosin. Casein behaves in a very similar manner, and is actually salted out, even at very low salt concentrations, on the acid side of its isoelectric point (Linderstrøm-Lang and Kodama, 124; Sørensen and Sládek, 201). Green (80) found very marked similar effects in horse hemoglobin solutions. Recent studies by M. H. Blanchard, J. L. Oncley and others in this laboratory have shown that the same is true for human serum  $\gamma$ -globulin (see Part VI), thus confirming and extending Hardy's early observations.

Solubility curves for proteins as a function of pH and ionic strength thus fall into two general classes—those of proteins like  $\beta$ -lactoglobulin, in which solubility rises on either side of the isoelectric point, and in which the pH of minimum solubility changes only very slightly with change of ionic strength; and those in which increase of ionic strength actually decreases solubility on the acid side of the isoelectric point, while increasing it on the alkaline side. This effect has, as its natural consequence, very marked shift of the pH at minimum solubility toward the acid side as the ionic strength increases\*.

\* As yet, there are no well authenticated cases of proteins in which increase of ionic strength decreases solubility on the *alkaline* side of the isoelectric point. Cer-

Unfortunately, it is not yet known how the composition of the solid phase varies with pH in systems of the latter type. It appears quite probable that, at least on the acid side of the isoelectric point, the solid phase must consist, not of isoelectric protein, but of protein cation combined with the anion of the added acid. The ratio of protein to anion may vary progressively as the pH decreases. On these points, unfortunately, our information is still most inadequate. However, the wide difference between different types of proteins in the form of the solubility curves at different pH and ionic strength provides a very great range of possible conditions which may be chosen in order to attain maximum effectiveness of separation.

#### 8. *Interaction Between Different Protein Anions and Cations*

Salt formation is possible, not only between proteins and the anions of an added acid, but also between different protein molecules carrying net charges of opposite sign. Thus, if a precipitation is carried out at a pH value intermediate between the isoelectric points of two different protein components of the system, the solid phase may consist, largely or wholly, of an insoluble salt made up of the anion of one protein and the cation of the other. The formation of the insoluble protamine-insulin complex from positively charged protamine and negatively charged insulin is perhaps the best known case of this phenomenon. The importance of avoiding such complexes, in an effective fractionation process, was perhaps first clearly stated by Green (82) who emphasized that all separations should take place, so far as possible, under conditions at which all the protein components present carry a net charge of the same sign, either positive or negative. This has been a guiding consideration in the planning of most of the fractionation processes described in Part VI.

#### 9. *Effects of Small Concentrations of Non-Protein Organic Anions and Cations*

In addition to hydrogen ions and simple inorganic anions and cations, larger ions undergo important and specific interactions with many proteins. These interactions are discussed in detail in Part VII. As yet, little is known about the effects of these substances on protein solubility. There are indications, however, that small amounts of certain organic anions and cations may, in some cases, produce disproportionately large changes in the solubility of proteins. Such effects must always be contained effects of this sort have been reported at extremely low ionic strengths, but it is probable, in such cases, that a true equilibrium was not reached between the protein in the liquid and in the solid phase at such extremely low salt concentrations.

sidered as possibilities; and salts containing such ions, even when present in very small amount, must be regarded as components of the system in the thermodynamic sense. Unfortunately, the effects are often so highly specific that few rules can be laid down in advance to determine whether a given substance is or is not a significant component. It is therefore necessary to be constantly vigilant and to take into account all possible contaminants whose presence may significantly modify solubility\*.

#### 10. *Separation of Certain Proteins by Adsorption*

The use of adsorption techniques in the separation and purification of proteins has hitherto been relatively limited. Such techniques were, of course, very extensively employed by Willstätter and his collaborators in the purification of enzymes, but did not lead to the separation of proteins which could be recognized as chemical individuals. Among the blood plasma proteins, prothrombin is particularly characterized by the readiness at which it is adsorbed by any of a large number of agents. This property of prothrombin has been used to great effect in achieving its purification, especially by Seegers, Loomis and Vandenberg (193), who have particularly employed adsorption on magnesium oxide as a major step in purification. Laki (121) has reported the use of tricalcium phosphate gel as an adsorbing agent in the purification of fibrinogen, and claims to have obtained crystalline fibrinogen by a process in which this adsorption is a fundamental step. However, his observations have not yet been confirmed in other laboratories and they clearly call for further investigation.

#### 11. *Effect of Temperature on Solubility: Heat of Solution*

The variation in solubility of a slightly soluble substance with temperatures is given by the thermodynamic relation :

$$\frac{\partial \ln S}{\partial T} = \frac{\Delta H}{RT^2} \quad (11)$$

Here  $\Delta H$  is the heat *absorbed*, at constant pressure, when one mole of solute passes into solution. If heat is *evolved* on solution, Equation (11) shows that the solubility will decrease with rising temperature. This case

\* The importance of many organic reagents, such as detergents, in the process of protein denaturation is, of course, well known. In the present connection, however, we are stressing the possible significance of very low concentrations which may not produce appreciable denaturation but may still markedly affect solubility.

† In Equation 11, it is assumed that the activity coefficient of the solute is independent of its concentration. If this assumption is not applicable, a more general type of equation must be used (see Cohn and Edsall, Ref. 39, p. 188-189)

is exceptional, but is by no means unknown in protein systems, especially for certain proteins in the salting out range. Thus Green (80) showed that the solubility of hemoglobin in concentrated phosphate buffers is decreased approximately 10-fold by a rise in temperature from 0 to 25° C., and Edsall (56) found that the solubility of myosin in concentrated salt solutions decreased with rising temperature to an even greater extent.

On the whole, however, the usual relation—absorption of heat on solution and increase of solubility with temperature—is found much more commonly in protein systems, especially at low ionic strengths. Different proteins often differ markedly in heat of solution. The classical method of preparation of edestin involved its crystallization by the gradual cooling of a very warm salt solution of the protein. Marked differential effects of temperature on solubility have been observed for different components among the globulins of normal plasma, notably for  $\gamma$ -globulin, and fibrinogen, and have been used already in certain procedures, especially for the purification of fibrinogen (Part VI).

#### IV. SEPARATION OF EUGLOBULINS IN AQUEOUS MEDIA AT LOW IONIC STRENGTH

We may now consider the fractionation methods most generally applicable to the separation of the plasma proteins. The discussion in the present section deals only with one class of proteins—the euglobulins, which are insoluble at their isoelectric points at sufficiently low ionic strength, but dissolve at higher ionic strengths, or at higher or lower pH values\*. Part V deals with the separation of proteins by salting out, and Part VI with the selective precipitation of proteins, at low temperature and low ionic strengths, by water-miscible organic precipitants such as

\*The term "euglobulin" has not always been used in this sense; frequently it has been used to denote proteins precipitated by 0.33 saturated ammonium sulfate solution. The two definitions are far from identical, as will become apparent in the subsequent discussion. Moreover, some authors have denoted as euglobulins only those proteins precipitated by removal of salt at some particular pH—commonly at or near pH 7. This definition is much more restricted than that adopted here; the prothrombin fraction of plasma, for instance, is very insoluble at low ionic strength at pH 5, but is soluble in the complete absence of salt at pH 7. We shall consistently term such proteins "euglobulins" in the present discussion.

The general term "globulin" has been given at least three different meanings: (1) to denote only euglobulins, as defined in the text here, (2) to denote proteins precipitated by half-saturated ammonium sulfate, (3) to denote proteins with an electrophoretic mobility lower than that of albumin. No one of these definitions denotes exactly the same class of proteins as either of the others; it is therefore important, when the term "globulin" is employed, to be clear as to the operational procedure which gives it meaning in any particular context. *Mutatis mutandis*, similar comments apply to albumin.



ethanol or acetone. These methods, employing organic precipitants, may indeed be regarded as a more generalized form of the methods for separation of euglobulins discussed in the present section; for, by decreasing the dielectric constant of the solvent, the pseudoglobulins and albumin may be brought to behave, at low ionic strengths, similarly to the euglobulins in water.

It has been known for a very long time that at least one class of globulins may be separated from whole plasma or serum by acidification and dilution. The classical method for carrying out this separation generally involved a ten to twenty-fold dilution of the plasma with water, accompanied by the addition of acetic acid. Early investigators learned to choose empirically the correct amount of acetic acid to give optimum precipitation. Later work showed that pH values in the neighborhood of 5.2 are particularly favorable for the separation of many of these euglobulin components. No attempt will be made to review the many early observations here; the two classical papers of Hardy (88) (see also 89) and Mellanby (144), published simultaneously in 1905, brought all of the earlier work into focus, and provided the foundation of a rational approach to the interaction of serum globulin with salts, acids, and alkalis.

Hardy, having formulated for the first time the conception of the isoelectric point, made an especially searching study of the solvent effect of acid and alkali on globulin, and on the properties of the resulting protein anions and cations. Mellanby's work was primarily concerned with the solubility of isoelectric globulin in salt solutions, and his paper is extraordinary as representing the first explicit formulation of the ionic strength principle in the words, "Solution of globulin by a neutral salt is due to forces exerted by its free ions. Ions with equal valencies, whether positive or negative, are equally efficient, and the efficiencies of ions of different valencies are directly proportional to the squares of the valencies" (144, p. 373).

Another 16 years elapsed before the generalized principle of the ionic strength, as describing the interaction of one electrolyte with another, was stated by G. N. Lewis (123). Actually, it must be noted that the solvent effects studied by Mellanby, although very striking and quite unmistakable, did not represent determinations of the true solubilities of individual components. He noted, in fact, that the amount of protein dissolved in a solvent of fixed composition was very nearly proportional to the total amount of undissolved protein added to a given volume of solvent. This shows plainly that the globulin fractions separated by him were by no means true individuals, but actually represented a complex mixture of many components.

A further and more searching attempt to fractionate such mixtures, and obtain pure individual components, was made in a long series of researches by Sørensen (198, 199), beginning shortly after 1920. In spite of prolonged and systematic attempts at fractionation, Sørensen was never able to obtain a serum globulin fraction which behaved as a single chemical individual. Indeed, it was largely the complexities which he encountered in the study of the serum globulins and albumins which led him to formulate his well-known theory of proteins as reversibly dissociable component systems (199). The history of this important concept and the criticisms which have been offered concerning it can not be discussed here\*.

The proteins identified as euglobulins by the procedure here described are only a small fraction of the total protein of serum, and indeed represent less than half of the globulin fraction, as separated by the salting out procedures described in the next section, or as identified by the electrophoretic or ultracentrifugal techniques already discussed. These euglobulins, however, do include certain components which are of major importance, because of their specific functions. Thus, Mellanby (146) found prothrombin to be a euglobulin readily precipitable at low ionic strength at pH values near 5.3, and his work has served as a starting point for all later processes for preparing prothrombin (193, 59). Likewise, the component  $C'_1$  (midpiece) of complement is also separated under almost identical conditions (Pillemer, 169); as is plasminogen, the inactive precursor of the proteolytic enzyme of plasma (Milstone 150; Christensen and MacLeod, 33). These components are further discussed in Part VI.

The most careful of the early workers in this field (Chick, 29; Michaelis and Rona, 149) had been led to the belief that the isoelectric point of serum euglobulin was in the pH range 5.0 – 5.5. This was indeed, in general, the region of maximum precipitation of globulin in the absence of salt. In 1926 and 1928, Felton (63, 64, 65) reported that he had obtained two fractions of serum globulin from anti-pneumococcus horse serum by precipitation at different pH values. One of these precipitated near pH 5 at low ionic strengths and was less soluble in dilute salt solution than the other, which precipitated near pH 6.8. The latter fraction contained the antibodies. Although some doubt was felt by other workers about Felton's conclusions at the time, later developments have abundantly justified his claim of the existence of globulins with isoelectric points well above pH 6. Reiner and Reiner (176) separated water-insoluble serum globulin into two distinct fractions; one precipitated most

\* For a brief critical review, see for instance, Cohn and Edsall (39, Chapter 23).

readily near pH 5 and the other above pH 6. This work was extended still further by Green (82), who separated three euglobulin fractions, which she recognized as distinct from normal horse serum. Two of them, denoted by her as  $P_I$  and  $P_{III}$  respectively, had a zone of maximum precipitation near pH 5 at low ionic strength. However,  $P_{III}$  was much more insoluble under these conditions than  $P_I$  so that it commonly precipitated, even at pH 6.3, along with the third fraction which was denoted as  $P_{II}$ . The latter, however, had a minimum of solubility at or near pH 6.2; it was found to be considerably more soluble at pH 6.2 than  $P_{III}$  at pH 5. By making use of these characteristic solubility differences, the three fractions could be separated.

These different fractions were found to differ distinctly in acid and base binding capacity, and the difference in the number of positively and negatively charged groups could be correlated with the physical properties of the different fractions.

In the process of separation of these fractions, Green emphasized the importance of making the separations under conditions in which all the components carried either a positive or a negative charge, so as to avoid formation of insoluble complexes between components carrying opposite charges (see Part III, p. 427). While the fractions separated by Green have later proved to be mixtures, these investigations revealed important distinctions among different classes of normal serum euglobulins, which served as an important guide to future work. The detailed discussion of the properties of these and other globulins is given in Part VI.

#### V. THE FRACTIONATION OF PROTEINS BY SALTING OUT\*

The fundamental principles of the salting-out method have already been discussed in Part III. In its application to proteins, only a few salts have been widely used for this purpose. The great majority of investigators have employed ammonium sulfate. The reasons for this choice have been twofold. On the one hand, as may be seen by inspection of Figs. 3 and 4, the salting-in action of ammonium sulfate at low ionic strengths is considerably less than that of sodium, potassium or calcium chloride, and the salting-out effect of sulfates is in general far greater than that of the corresponding chlorides. Full advantage can be taken of this high salting-out effect, because of the very great solubility of ammonium sulfate in water, which permits the attainment of extremely high ionic strengths. Thus a much greater range of conditions for salting-out is

\*The history of these methods is long, complex, and very interesting; but no attempt will be made to present a historical survey here. An excellent brief historical survey has recently been given by Pedersen (166, pp. 52-57 incl.). For earlier discussions, with references, see for instance Howe (108) and Cohn (35).

attainable than with any of the other salts that have been employed. These advantages of ammonium sulfate are partly counterbalanced by certain drawbacks inherent in its use. On the one hand, the presence of nitrogen in the form of ammonium ion renders impossible the direct use of nitrogen analyses on the solution in order to determine the protein concentration. To carry out such analyses, the salt must be removed by dialysis, or the protein heat coagulated or separated in some other way. Moreover, ammonium sulfate is not a good buffer, in the pH range below 8, where it is commonly employed, and the pH of the solutions ordinarily used, even if the salt has been carefully purified, is very sensitive to small additions of acid or alkali.\* In general, it is important, if satisfactory results are to be obtained, that the pH of the solution should be determined, and adjusted to the particular value regarded as most satisfactory for the experiment in hand†.

It is also to be noted that the usual procedure of expressing concentrations in terms of the fractional saturation of the solution with ammonium sulfate is sometimes ambiguous. The temperature coefficient of solubility is considerable for these solutions, and unless the temperature of the measurements is specified, the given conditions may be difficult for other investigators to reproduce exactly. For temperature values in the neighborhood of 20 to 25°C. half saturated ammonium sulfate may be taken as equivalent to a 2.05 molal solution. Other fractional saturations may be calculated by proportionality (see also 43).

Sodium sulfate has been perhaps more widely used than any other salt except ammonium sulfate, as in the studies of Howe (107). It may be readily seen by referring to Figs. 3 and 4, that the salting-out action of sodium sulfate at any given ionic strength is greater than that of ammonium sulfate. However, sodium sulfate is a much less soluble salt, and the range of conditions attainable with its use is therefore more limited.

\*Pure ammonium sulfate, being a salt of a weak base and a strong acid, when dissolved in water, gives a pH distinctly acid to neutrality. However, the pH may be adjusted to the acid side by adding  $\text{H}_2\text{SO}_4$ , or to the alkaline side by adding  $\text{NH}_3$ , without introducing ions not already present in the system.

†pH measurements should not be made directly on the concentrated ammonium sulfate solution, for the large salt error will render indicator measurements unreliable, and the high liquid junction potential will make electromotive forces uncertain of interpretation. It is therefore to be recommended that concentrated ammonium sulfate solution should be diluted previously to 0.3 M, or below, before testing the pH. Since these solutions are not very good buffers, this procedure involves some uncertainty, but it appears to be more reliable than the direct attempt to measure the pH of a concentrated salt solution.

The use of concentrated potassium phosphate buffers for salting-out was introduced after the extensive studies of activity coefficients in this system by Cohn (34) (see also Green, 81). They were used, for instance, by Florkin (73) in the separation and characterization of fibrinogen from horse plasma, and later by Butler and Montgomery (23) (see also 24) who used it systematically for the separation of plasma into fractions. Wuhrmann and Wunderly (219) have employed this method in a very extensive series of studies on pathological plasmas. Since the present review is concerned with the proteins of normal plasma, no attempt will be made here to review their monograph but it should be mentioned here for the wealth of information which it contains.

Some of the advantages of the potassium phosphate buffer system are obvious. The pH is well defined and can be stabilized at any given value, over a wide range of ionic strength. The salting-out constant for the phosphates is very great, being even larger than that for ammonium sulfate. However, it should be pointed out that the measured pH changes quite rapidly with dilution, so that if a concentrated phosphate solution is made up, using a given ratio of primary to secondary phosphate, its pH changes progressively as it is diluted with water\*. Moreover, the solubility of either sodium or potassium phosphate buffer mixtures is not sufficiently great to attain ionic strengths as high as those that can be reached with ammonium sulfate. For these reasons the use of phosphate in salting-out, in spite of its great advantages, has not yet become widespread.

Sodium chloride can be used to separate some of the proteins most readily salted out from those which require higher ionic strengths than are attainable with its use. The classical example is the use of half saturated sodium chloride solution to precipitate fibrinogen from plasma, a procedure first employed by Hammarsten (87). Potassium chloride is, in general, less suitable than sodium chloride, because of its lower solubility which limits the ionic strengths attainable by its use.

Sodium citrate buffers have been employed by Jameson and Alvarez-Tostado (109) for the fractionation of plasma. They report very favorable results from the use of these buffers and claim them to be superior in many ways to phosphate or ammonium sulfate. However, the fractionations reported by them are only preliminary, and they did not

\* It should be noted that the published pH values by Cohn (34) and the nomograms given by Green (81) refer to the potassium phosphate buffer system. If sodium phosphate buffers are used instead, the pH values at high ionic strength are very significantly different. Therefore, if concentrated sodium phosphate buffers are to be employed, their pH should be checked by the investigator, since comprehensive data on these systems are not available.

separate any fractions with a high degree of homogeneity, as judged by electrophoretic measurements.

A few other salts have been employed occasionally in salting-out studies, but their use has been so rare and irregular that they require no further discussion here. The rest of this section will deal primarily with the results obtained by ammonium sulfate fractionation, and the use of other salts will be mentioned incidentally as the discussion proceeds.

The traditional description of ammonium sulfate fractionation divided the plasma proteins into fibrinogen, separated at approximately .20-.25 saturation with ammonium sulfate; euglobulin, separated by .33 saturation; pseudoglobulin, separated at .50 saturation, and albumin which remains in solution after all the other fractions have been precipitated. It has long been recognized that this simple scheme of procedure did not sharply separate a series of homogeneous and uniform fractions; and reprecipitations of various sorts have always been employed by those investigators, such as Chick and Martin (30), who sought to obtain fractions of a high degree of purity. The work of the most careful investigators has made it apparent that these attempts, except in the case of the albumin fraction, were almost uniformly unsuccessful. Sørensen (198, 199), for instance, carried out a long and elaborate series of studies on serum euglobulin and pseudoglobulin fractions. His fractionation methods were varied and painstaking, and the products obtained were studied with the utmost care. The results showed clearly, however, that none of the globulin fractions obtained behaves even approximately like a single component in solubility experiments; this work has already been briefly discussed in Part IV. Sørensen's studies on serum albumin, carried on at the same time, also demonstrated that none of his preparations could be regarded as single components by the solubility test, although the deviation of the albumins from this behavior was not nearly so great as that of the globulins.

Among the serum albumins of different species, there are marked differences in carbohydrate content. The crude albumin fraction from horse serum, as prepared by salting-out, contains considerable amounts of carbohydrate, while that from human or bovine serum contains comparatively little. The fractionation of the serum albumins from horse serum has been studied in several laboratories (Sørensen and Haugaard, 200; Hewitt, 98, 99, 100; Kekwick, 113; McMeekin, 137, 138). All of these workers obtained a crystalline carbohydrate-free albumin fraction from horse serum, but the yields were generally small and large quantities of carbohydrate-containing material were left in the filtrates. From such filtrates Hewitt (100) isolated a globulin fraction with a carbohydrate

content of 4.6 and a fraction which he called seroglycoid containing 8.6 per cent of carbohydrate; neither of these two latter fractions was obtained in crystalline form, and their properties differed markedly from those of the crystalline albumin. Kekwick likewise obtained a crystalline fraction containing 1.95 per cent carbohydrate; and McMeekin, by a painstaking fractionation procedure, obtained a crystalline fraction containing approximately 5.5 per cent carbohydrate. This fraction differed in crystalline form, in nitrogen content, and in optical rotation, from the crystalline carbohydrate-free albumin of horse serum.

In these separations, McMeekin added the ammonium sulfate used for crystallization by dialysis through a rotating cellophane bag immersed in the protein solution. The amount of salt initially placed in the bag was calculated so as to give the desired final concentration of ammonium sulfate in the whole system at equilibrium. In this way, the increase of salt concentration with time was very gradual and conditions for crystallization were more favorable than when the salt was added rapidly.

Cohn, McMeekin, Oncley, Newell, and Hughes (43) made systematic studies of different fractions obtained by ammonium sulfate precipitation. They found, using horse serum, that 14 out of the total of 72 g. of protein per liter of serum were precipitated at .33 saturation, and the precipitate consisted almost entirely of  $\gamma$ -globulin. Between 0.34 and 0.40 saturation, a mixture of  $\gamma$ - and  $\beta$ -globulins was precipitated, and practically no  $\gamma$ -globulin remained in the filtrate from this precipitate; between .40 and .50 saturation, a mixture of  $\beta$ - and  $\alpha$ -globulins was precipitated, together with a trace of albumin. The fractions precipitated at higher ammonium sulfate concentrations consisted of albumin together with other important

TABLE VII  
*Serum Proteins of the Horse*

Moles per Liter	Concentration of Ammonium Sulfate		Type of Protein Precipitated	Per Cent of Total Protein Pptd.	Per Cent of Protein Pptd.	
	Per Cent of Satn.				Water- Soluble	Water- Insoluble
1.39	0.34	Largely $\gamma$ -globulin		20	71	29
1.64	.40	$\gamma$ -, $\beta$ -, $\alpha$ -globulin		15	67	33
2.05	.50	$\beta$ -, $\alpha$ -globulin, mucoglobulin		14	94	6
2.57	.62	Largely crystalline albumins		32	98	2
2.80	.68	Crystalline albumins, hemo- cuprein, choline esterase, glycoprotein, phosphatase		14	99	1

From Cohn, McMeekin, Oncley, Newell and Hughes (ref. 43).



components present in only small amounts, such as serum esterase, and hemocuprein (concerning the latter, see Mann and Keilin, 140).

A further purification of several of these initial fractions was undertaken and  $\gamma$ -globulins of quite high purity were obtained. The purification of  $\alpha$ - and  $\beta$ -globulins was not carried so far. Sedimentation and diffusion constants and electrophoretic mobilities were reported for the various fractions; these will not be discussed here, since the results of similar but far more extensive studies on fractions more highly purified by a different method are given in Part VI.

The most extensive recent series of fractionation studies by salting-out with ammonium sulfate is that of Pedersen (166). His studies on the X-protein of plasma, and on fetuin from fetal sera, have already been discussed in Part II. In addition, he carried out a very long and extensive series of studies on the fractionation of normal plasmas of various species, varying the ammonium sulfate concentration systematically and, in many cases, removing fractions at various steps, sometimes at pH 5 and sometimes at pH 7. Other pH values were not, in general, employed in his work. The resulting fractions were studied primarily in the ultracentrifuge; a few data on electrophoretic mobility are recorded for some of the experiments. For the most part, these studies did not yield individual fractions of a high degree of homogeneity and they will, therefore, not be described here in further detail. Pedersen's work, however, should be consulted, for the wealth of detailed information that it contains, by all who are seriously concerned with the fractionation of serum proteins by the salting-out method.

#### VI. LOW TEMPERATURE FRACTIONATION OF PROTEINS AT LOW IONIC STRENGTHS IN THE PRESENCE OF WATER-MISCIBLE ORGANIC PRECIPITANTS

The discussion of the fundamental principles of fractionation (Part III) has shown that proteins are most sensitive to the action of electrolytes, at low ionic strengths and in media of low dielectric constant. The solvent action of salts at low ionic strengths is profoundly influenced by the dipole moment, and the quadrupole and higher electric moments, arising from the distribution of positive and negative charges within the protein molecule and over its surface. These electric configurations are often highly characteristic of individual protein molecules, for some of which great changes in solubility can often be effected with small variations in the ionic strength or dielectric constant of the solvent. Furthermore, both the effects of salt at low ionic strength and the effects of such organic reagents as ethanol are highly specific for different individual proteins. In contrast, the salting-out effect in highly concentrated salt



solutions is relatively insensitive to the specific chemical characteristics of the protein, and depends very much on such comparatively unspecific factors as the general size and shape of the molecule. The salting-out process involves a system which may be described in terms of four essentially independent variables, namely, salt concentration, protein concentration, pH, and temperature.\* On the other hand, if fractionation is carried out by the addition of ethanol at low temperatures and low ionic strengths, an additional independent variable is introduced, namely, the ethanol concentration. The presence of even one additional variable in such a complex system enormously increases the range of possible conditions which can be chosen for the separation of any given component.

For large scale fractionation of proteins, the low salt-ethanol method has another advantage over the salting-out procedures. In the latter, the high salt concentrations necessary for protein precipitation can be diminished only by dialysis, a procedure which is time consuming and involves much work when a large volume of liquid is to be dialyzed. Furthermore, during the later steps of dialysis when the salt concentration is diminished, conditions become favorable for bacterial growth, and the protein solution is likely to become contaminated. If ethanol or acetone is used as a precipitant, instead of salt, it may be readily removed because of its volatility. By far the safest method for such removal appears to be the rapid freezing of the solution at very low temperature with subsequent drying from the frozen state in a vacuum. The cooling produced by evaporation maintains the frozen protein solution at low temperature through almost the whole of the process, and the temperature begins to rise only when the protein is nearly dried. Under these

\*The effect of variation of pressure on protein solubility may well be important, but it seems extremely unlikely that pressure can be utilized as an additional variable in protein fractionation. The use of sufficiently high pressures is known to denature many proteins, and the maintenance of more moderate pressures up to perhaps two or three hundred atmospheres would scarcely be technically feasible under the conditions necessary for separating protein fractions as they precipitate.

For simplicity, we have spoken here as if protein concentration were a single variable. Properly speaking, of course, there are as many variables involved as there are independent protein components in the system. The actual number of components in such a system as blood plasma is certainly very large, and is in general unknown. In speaking of protein concentration as a variable, of course, it is implied that we are considering, in any given step of fractionation, a mixture of proteins in more or less constant proportions. In the successive steps of an elaborate fractionation process, the ratio of the different protein components is markedly altered, as some components are largely removed from the system during the earlier steps of fractionation. In speaking of protein concentration as a single variable, we are using a shorthand description whose full meaning is to be interpreted in the sense explained here.

circumstances, most of the plasma proteins, and a great many other proteins, are found to be completely soluble in water or dilute salt solutions after drying is complete, and they may be preserved in the dry state without alteration for long periods of time. Certain proteins indeed—in blood plasma these include particularly the lipoproteins—can not be dried without denaturation, and for their purification dialysis may be necessary at certain steps in the procedure. However, serum albumin, fibrinogen, and most of the globulin components of plasma can be dried from the frozen state without impairing their solubility or their specific properties.\*

Combined with these advantages, the method also has certain definite dangers. Although variation in temperature can often be used to separate certain components with a large heat of solution, the upper limit of the temperatures employed in such a system must be low, in order to avoid denaturation of the protein. In the system of plasma protein fractionation employed throughout the war at the Plasma Fractionation Laboratory, the temperature at every step has been held at or below 0°C. As soon as sufficient alcohol has been added, the temperature is reduced below 0°C.; and in all steps at which the alcohol concentration is greater than 20% by volume, the temperature is maintained at or below -5°C. Special precautions are needed to insure that the temperature is held at these assigned levels at every stage during the process. The mixing of alcohol and water is attended with evolution of heat. It is therefore important, not only that the ethanol addition should be carried out in cold surroundings, but it is further necessary to insure additional cooling during the mixing of ethanol and water. If the process is carried out on a small scale, this may be done by immersing the container in a low temperature bath, and stirring constantly to attain temperature equilibrium between the liquid in the container and the bath outside. Containers made of stainless steel are advantageous for this purpose because of their high heat conductivity. When large scale processing is carried on, the precipitations are usually carried out in large glass lined tanks in a cold room. Cooling during the addition of ethanol can be achieved in such cases by the circulation of refrigerant through a spiral coil of stainless steel tubing, immersed in the plasma solution. Here again, constant stirring is necessary during the addition of ethanol, which must in all cases proceed slowly, usually by inflow through capillary tubing. Rapid stir-

\*The technique of drying proteins from the frozen state cannot be discussed here for lack of space. The following reference may be consulted: Florsdorf and Mudd (74), Florsdorf, Stokes and Mudd (75), Greaves and Adair (79), Hill and Pfeiffer (102), Strumia, McGraw and Reichel (207), Strumia and McGraw (206).

ring is essential here in order to prevent any element of the protein solution from attaining, even temporarily, an unduly high ethanol concentration which might denature the protein.

These precautions concerning temperature and the method of the addition of ethanol are of major importance. It has been found by experience in this laboratory during processing that even a brief rise of temperature to a few degrees above  $0^{\circ}$  for a few minutes may have definite and undesirable effects on the stability of the end product obtained by fractionation. While it is true that certain proteins appear to resist exposure to considerable ethanol concentrations, even at room temperature, with little or no sign of denaturation, it must still be considered that such proteins are the exception rather than the rule. Even when denaturation is undetectable by most tests, sufficiently careful observations may indicate that some of the protein molecules have become altered. Rigorous precautions should be taken to insure the maintenance of low temperatures, in any protein system involving such reagents as ethanol or acetone, until clear and definite proof has been obtained that the proteins under investigation can be safely precipitated in such media at a higher temperature. The necessity for these precautions in fractionation by the low temperature, low salt, ethanol method makes it in many respects a more difficult process to carry out than is fractionation by salting-out with ammonium sulfate. The necessary precautions are fundamentally simple, but they must be strictly maintained and not relaxed in any step in the process. Once the proper design of apparatus and equipment has been obtained, however, and provided the workers operating the process understand the necessity for these precautions, it is possible to carry through a quite elaborate fractionation procedure with a group of workers with a relatively rudimentary knowledge of science and technology, provided that their director be a competent supervisor. This has been abundantly proved by the success of the large scale fractionation of blood plasma during the war, utilizing these methods in a group of seven different pharmaceutical houses.

### 1. *Choice of Conditions in the Fractionation of Plasma*

Alcohol, acetone, ether, and other related reagents had, of course, been constantly employed, beginning early in the nineteenth century, by protein chemists to precipitate proteins, to wash them free of impurities, and prepare them for analytical study. For the analytical chemist the accompanying denaturation of the protein was not generally a matter of concern, provided no small fragments of the native protein molecules were split off and washed away by the treatment, before analysis was

begun. For preparation of undenatured proteins by such reagents, however, precautions of the sort described above were essential, and these were seldom taken. One of the first relatively successful uses of alcohol in this connection was the concentration of diphtheria antitoxin by ethanol precipitation in the cold, as employed by Mellanby (145) in 1908. This was also one of the earliest publications in which the protein nature of an antibody was definitely claimed. Two years later, Hardy and Gardiner (90) published a brief account of experiments of fundamental importance, involving the precipitation of plasma proteins with ethanol (or acetone) at low temperature, and their subsequent washing with ethanol and ether to remove lipids. The resulting dried protein preparations readily redissolved to give clear and stable solutions in water or physiological saline. The achievements of such dried preparations represented a major advance in protein chemistry; however, later studies with the ultracentrifuge have revealed that the removal of lipids by the alcohol-ether techniques is accompanied by marked changes in the sedimentation diagram (McFarlane, 132; Pedersen, 166, especially pp. 43-51 incl.). The alterations in the lipoproteins of native plasma which this treatment must produce would be expected to be drastic, in the light of our present knowledge, so that these findings are not surprising.\*

Wu (218) successfully employed low temperature-ethanol precipitation in the preparation of undenatured proteins; and Ferry, Cohn and Newman (71, 72) employed ethanol-water, at  $-5^{\circ}$  C., at low ionic strengths, as a medium for studying the solubility of egg albumin and horse hemoglobin in solvents of low dielectric constant. They showed that the solvent action of neutral salts under these conditions was much greater than in water; and that both these proteins could be successfully recrystallized, after exposure to 15-25 per cent ethanol at  $-5^{\circ}$  for long periods, provided the ethanol was removed before the temperature was raised.

The first report on a systematic fractionation of plasma by the low temperature — low salt — ethanol procedures was made in 1940 by Cohn, Luetscher, Oncley, Armstrong, and Davis (41). During the war, these methods were classed as confidential, and a very long series of plasma fractionation runs was made, using human blood, collected by the American Red Cross, in connection with the development of plasma fractionation products for use by the Armed Forces.† Much information

\* Concerning the lipoproteins of plasma, see p. 457.

† This work was originally supported by grants from the Rockefeller Foundation and from funds of Harvard University. It was aided early in 1941 by grants from the Committee on Medicine of the National Research Council, which included a grant from the American College of Physicians. From August 1941 to July 1946

concerning the nature of the products obtained, and their clinical uses, was released during the war,\* but the first detailed report of the methods used was given in 1946 by Cohn, Strong, Hughes, Mulford, Ashworth, Melin and Taylor (45). An earlier report (44) (see also 217) gave many details concerning the properties of the plasma fractions.

The methods of plasma fractionation, as developed at the present time, represent a gradual evolution, based on the attempt to develop an inclusive process which should yield all the components of plasma in active, concentrated and undenatured form. This goal, of course, is still far from achievement, but the most recent processes have represented a great advance over the earlier ones, both with respect to the number and specificity of the active fractions separated, and with respect to the preservation of components in undenatured form.

Certain properties of the protein components of human plasma have been recognized, as experience was accumulated, and formed the basis of the plasma fractionation process employed at present. Some of them may be stated as follows: (1) Most of the fibrinogen is readily precipitated at very low ethanol concentrations (8 to 10% by volume), at temperatures between 0° and -3°C., and pH values near 7. The fraction so obtained is known as Fraction I. (2) Virtually all the  $\gamma$ -globulin, and a large portion of the  $\beta$ -globulin, including the  $\beta_1$ -lipoprotein (X-protein), is precipitated on raising the ethanol concentration to 25% by volume, while lowering the temperature to -5°C. at pH near 7. This fraction, designated as II+III, also contains prothrombin and the isoagglutinins. These various components are separated by methods described later. (3) A large portion of the  $\alpha$ -globulin, much of it a lipoprotein containing about 35% lipid (cholesterol, fatty acid, phosphatide), can be separated from the supernatant of Fraction II+III by lowering the ethanol concentration to 18% and the pH to 5.2. This fraction is denoted as IV-1. Another component of this fraction is a blue-green pigment, previously described by Luetscher (130) and others. In the earlier procedures employed for fractionation (see 45) the  $\alpha$ -lipoprotein was largely denatured, and the lipid removed from its attachment to the protein component, by carrying out the precipitation at higher alcohol concentration (40%) and pH 5.5-5.8. It was found that denaturation could be

it was carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

\* See particularly a series of papers in the *Journal of Clinical Investigation* 23, 417-606 incl. (1944); 24, 657, 662, 671, 698, 704, 793, 802 (1945). Many of these papers are referred to separately in the bibliography.

avoided if the protein in the fraction was never exposed to ethanol concentrations higher than 25%, its precipitation being readily brought about in 18% ethanol because of its rapidly decreasing solubility with decrease of pH between 6 and 5.2. (4) The supernatant from Fraction IV-1 contains other  $\alpha$ -globulins, virtually lipid-free, certain  $\beta$ -globulins, and nearly all the albumin. To separate the globulins, while leaving the albumin in solution, the ethanol concentration is raised to 40% (0.163 mole fraction ethanol) and the pH to 5.8. The resulting precipitate (Fraction IV-4) contains some albumin and  $\alpha$ - and  $\beta$ -globulins. The latter include serum esterase and a specific iron binding globulin of plasma. (5) The protein in the supernatant from Fraction IV-4 is almost entirely albumin. It is now separated by lowering the pH to 4.8, with pH 4 acetate buffer,\* while maintaining the ethanol concentration at 40%. The albumin is practically isoelectric, under these conditions, and precipitates almost quantitatively as Fraction V. The albumin in the resulting precipitate contains about 4% of  $\alpha$ - and 1% of  $\beta$ -globulin, which are less heat-stable than the albumin. They can be largely removed by redissolving the precipitate in water, and adding ethanol to a volume concentration of 10% at  $-3^\circ$ , at pH 4.6 and ionic strength 0.01. The resulting small precipitate is filtered off, and the filtrate contains albumin of 97-99% purity, as judged from electrophoresis in pH 8.6 barbiturate buffer. The globulin content can be reduced to a few tenths of one per cent, or less, by crystallization of the albumin, as described later.

The supernatant from Fraction V, when concentrated by evaporation at low temperature, is known as Fraction VI. It contains a very small amount of albumin and  $\alpha$ -globulin (less than 2% of the total protein in plasma) large amounts of salt, and some of the low molecular weight non-protein substances found in plasma.

The conditions for separation of the principal fractions of human plasma, and the electrophoretic data concerning them, are summarized in Table VIII. The procedure for separation here described is known as method 6; it has evolved from a series of earlier methods described in detail elsewhere (45), and is obviously subject to further modification and improvement. Moreover, it should be emphasized that the method as described, and the data obtained, apply specifically only to human plasma. Plasma from other species invariably shows characteristic

\* The pH values are not measured directly on the solution of high ethanol content, but are determined on the glass electrode after diluting the solution with water to a final content of about 4% ethanol. Acetate buffer is used, rather than acetic acid, so that the protein solution is never exposed, even momentarily, to pH values more acid than 4. The preparation of acetate buffers, of a given pH and ionic strength, is conveniently made with the aid of the nomogram given by Boyd (17).

TABLE VIII  
*Separation and Electrophoretic Composition of Principal Fractions of Human Plasma by Method 6*

Fraction	pH	Conditions of Separation			Protein in System, g./l.	Electrophoretic Composition g. per Liter of Plasma Estimated in Plasma					Total
		V/2	Temp. °C.	Mole. Fraction Ethanol		Albumin	$\alpha$	$\beta$	$\gamma$	Fibrinogen	
Plasma	7.4	.16	...	...	60.3	36.3	9.2	10.6	7.2	2.5	65.8
I	7.2	.14	-3	.027	51.1	0.2	0.3	0.5	0.3	2.1	3.4
II+III	6.8	.09	-5	.091	30.0	0.8	1.1	9.1	7.0	1.0	19.0
IV-1	5.2	.09	-5	.062	15.8	0	4.5	0.5	0.1	0	5.1
IV-4	5.8	.09	-5	.163	10.1	0.9	2.7	2.2	0	0	5.8
V	4.8	.11	-5	.163	7.5	29.9	1.3	0.3	0	0	31.5
*VI	4.8	.11	-5	.163	0.2	0.8	0.2	<.1	0	0	1.0
Total	...	..	..	...	...	32.6	10.1	12.6	7.4	3.1	65.8

Data from Cohn, Strong, Hughes, Mulford, Melin and Taylor (45); see especially Tables II and IX of their paper. Electrophoretic figures in the top row are for measurements directly on plasma; those in the bottom row are summations from the distribution in the plasma fractions, multiplying by the appropriate factor for the percentage of total plasma protein represented in the fraction.

Electrophoretic components of plasma with mobilities close to that of fibrinogen are resolved with difficulty. The value of 2.5 g./l. for fibrinogen in whole plasma is derived from Edsall, Ferry and Armstrong (59), who base their estimates on the amount of protein elutable with thrombin (see also Morrison, 134). The remainder of the protein with the same mobility is included with the  $\beta$ -globulins. A similar situation occurs with Fraction II+III. Satisfactory resolution of the  $\alpha$ -globulin from albumin in Fraction IV-1 has not been accomplished, and since probably less than 25% of the fraction is albumin, any components with mobilities similar to that of albumin have been included with the  $\alpha$ -globulins. Further studies on this point are in progress.

differences (see for instance the electrophoretic data in Table IV) which will be reflected in the fractionation. The  $\beta_1$ -lipoprotein (X-protein of Pedersen (166) ) found in Fraction II+III, for instance, has been recognized only in human plasma; a protein of this type has been found only in traces, if at all, in the animal plasmas hitherto examined. The details of the plasma fractionation procedure may therefore require important modifications, depending on the species of plasma being fractionated.

For some purposes the principal fractions can be employed as such. For instance, Fraction I is a suitable starting material for the preparation of fibrin foam and film; and Fraction V, after a single reprecipitation, as already described, can be dissolved in a solution containing a suitable stabilizing agent, such as the sodium salt of acetyl-tryptophan\* at 0.04 M, to give a concentrated solution of high stability which has found extended clinical use. However for many purposes further subfractionation of the principal fractions is essential, in order to obtain separately the many important components they contain.

## 2. *The Subfractionation of Fraction I: Fibrinogen and the Anti-Hemophilic Globulin*

Fraction I as first separated from human plasma contains approximately 60 to 65% fibrinogen.† This factor is determined more specifically by measuring the amount of protein from the fraction which will form a fibrin clot with thrombin. To give reliable results for fibrinogen assay, the clot must be formed under suitably chosen conditions of pH, ionic strength, and fibrinogen and thrombin concentration. A systematic study of these factors has been made by Morrison (154).‡

Fraction I, although very far from being pure fibrinogen, has served as an excellent starting material for the preparation of many fibrinogen and fibrin products, such as fibrin foam and fibrin film (14, 68, 69, 70). However, for a deeper understanding of the clotting process, and for chemical studies of the fibrinogen molecule, a much more highly purified

\* See Part VII.

The fibrinogen content of bovine Fraction I is generally 75% or better, reflecting the much higher concentration of fibrinogen in bovine plasma (52, 104).

† Misleading results can, of course, be given if other plasma proteins are occluded in the clot as it forms. Morrison's studies have shown that the lipoprotein Fractions III-0 and IV-1 have especially high tendencies to occlusion; the non-clottable protein of Fraction I is also occluded more than most of the other plasma protein components. Albumin is virtually not occluded at all. Morrison's findings on occlusion have an obvious significance for the assay of fibrinogen in whole plasma, particularly in some pathological plasmas containing high concentrations of lipids and lipoproteins.



fibrinogen is essential. Recent studies (155) have shown that two characteristics of the protein in Fraction I are particularly important for this separation: (1) The solubility of fibrinogen decreases more rapidly with decrease in pH, between pH 5 and 7, than that of the non-clottable protein of Fraction I. (2) The temperature coefficient of solubility is very large, both for fibrinogen and for the non-clottable protein. However, there is one portion of the non-clottable protein with an extremely high temperature coefficient of solubility, which can be conveniently separated by lowering the temperature of a concentrated Fraction I solution from 10° or above to approximately 0°. A certain amount of fibrinogen is lost in this separation, but the residual more soluble material has a much higher fibrinogen content than the original Fraction I. Making use of these observations, human fibrinogen preparations containing 98% of clottable protein, or better, have been obtained.

Human fibrinogen is a very large elongated molecule with a length of the order of 700Å, as judged by double refraction of flow studies (60). Its sedimentation constant,  $s_{20}$ , is approximately 9 S as reported by both Oncley, Scatchard and Brown (162) (see Table XI) and by Holmberg (105). Holmberg estimates the molecular weight as near 700,000 from sedimentation and diffusion, while Oncley gives a figure of approximately 400,000, based on sedimentation, viscosity, and double refraction of flow. The preparations studied in the two laboratories were made by quite different methods, and further work is required to resolve the discrepancy in the estimated molecular weight.

Fraction I of normal plasma contains a high concentration of another component, at present recognizable only through its influence in shortening the clotting time of hemophilic blood, even when added at very high dilution. This factor is also present in Fraction III-2, 3 which contains prothrombin and plasminogen, but the concentration is far higher in Fraction I. Preparations of Fraction I, dried from the frozen state in sterile form, have been used clinically by a number of investigators to shorten clotting time in hemophilic patients who were about to undergo an operation, or who were suffering from internal bleeding. In many cases, the treatment has proved strikingly effective. The results are, of course, only temporary and are essentially identical with those which can be obtained by injections of compatible whole blood or plasma (152, 213). A few hemophilic patients, who respond to whole plasma, are not benefited by Fraction I injections.

As yet all attempts to separate the antihemophilic factor from fibrinogen by chemical means have proved unsuccessful. The two are clearly distinct; fibrinogen, for instance, is readily heat coagulated in 5 minutes

at 53°, whereas the antihemophilic activity of Fraction I is still high after this heat treatment, although it is rapidly lost at 65-70°C. Further research on the chemical nature or the antihemophilic factor is clearly necessary.

### 3. Subfractionation of Fraction II+III

This fraction contains virtually all the antibodies of plasma, as well as prothrombin, the isoagglutinins, plasminogen, and the component C<sub>1</sub> of complement. The separation of these various components is, of course, of major importance, and a systematic procedure has been developed by Oncley, Melin, Cameron, Richert and Gross (161). Briefly, it is based on the following observations:

1. The  $\beta_1$ -lipoprotein (X-protein) is soluble at pH 7.2-7.6 even at relatively high ethanol concentrations (17-20%), whereas nearly all other components of this fraction are insoluble under these conditions if the ionic strength is sufficiently low. The removal of the lipoprotein, as the first step in the subfractionation process, enables it to be separated without denaturation, and allows the subsequent separation of all the other components of Fraction II+III in virtually lipid-free condition.

2. Prothrombin, plasminogen, and the fibrinogen not already precipitated in Fraction I, are very insoluble at pH 5.2-5.4, even in the absence of alcohol and at moderately high ionic strengths. This fraction is denoted as III-2, or III-2,3.

3. The isoagglutinins are also insoluble at pH 5.2-5.4 at very low ionic strengths, but dissolve readily at this pH, on slightly increasing the ionic strength to a value insufficient to dissolve prothrombin, fibrinogen, or plasminogen. The pH of minimum solubility for the isoagglutinins is near 6.3; they are readily precipitated at this pH, at low salt and low ethanol concentrations, the resulting fraction being known as III-1.

4. Most of the  $\gamma$ -globulins are least soluble at pH values near 7. At pH 5.2-5.4 they are very soluble, probably more soluble than any other component of plasma at this pH and at low ionic strength. One portion of the  $\gamma$ -globulins (Fraction II-3) is precipitated in this pH region by a moderate increase of ionic strength; it thus belongs to the class of proteins which are salted out, even at low ionic strength, on the acid side of the isoelectric point (see discussion on p. 426). The remainder—the major part of the  $\gamma$ -globulin (Fraction II-1, 2)—is precipitated most readily near to, or slightly above, pH 7, at ethanol concentrations near 20% at -5°C.

5. Plasminogen is strongly absorbed on a fibrin clot, if the clot is formed in a solution containing plasminogen.

The steps in the subfractionation of Fraction II+III (161) will not be discussed in detail here, but are shown diagrammatically in Fig. 9.

To give some notion of details of procedure, the first step in the process shown in Figure 9 has been described as follows:

"Each kilogram of precipitate II+III is suspended in 2 kilograms of water containing ice (about one-quarter of the water should be frozen, in the form of very fine ice crystals). When this suspension is fairly uniform, add 3 kilograms of 0° water to which 112 cc. of 0.5 M  $\text{Na}_2\text{HPO}_4$  (pH 9.2) has been added. This suspension should be stirred slowly and kept at a temperature of 0° C. until all lumps are dissolved, and a nearly complete solution is obtained. After the suspension is complete, add it to 20 kilograms of 0° water, and stir slowly at 0° for 30-60 minutes. The pH of this suspension should read  $7.2 \pm 0.2$ . Then bring to 20% ethanol by adding 15 liters of 53.3% EtOH, keeping the temperature as low as possible until -5° C. is reached. This suspension should stand at -5° C. with slow stirring for several hours before centrifuging. Centrifuge at a rate of about 30 liters/hour.

"*Precipitate II+III* consists of practically all of the  $\gamma$ -globulins, isoagglutinins, and prothrombin originally present in the Fraction II+III. It contains very little cholesterol or carotenoid pigment. From a pool of normal plasma, it should represent about 60% of the original II+III paste, and will contain 50-55% of  $\gamma$ -globulin by electrophoresis at pH 8.6." (Description prepared by the Plasma Fractionation Laboratory, Harvard Medical School, dated July 27, 1945.)

Fraction III-2,3—the euglobulin fraction with minimum solubility near pH 5.2—contains prothrombin, the midpiece (component  $\text{C}'_1$ ) of complement, and plasminogen, the precursor of the proteolytic enzyme of plasma.

*Prothrombin and Thrombin.* Prothrombin is somewhat unstable at such an acid pH as 5.2, and this fraction therefore should be promptly redissolved at higher salt concentration and at a pH of 6 or higher. Human prothrombin, as obtained in this fraction, has been generally converted to thrombin by the action of calcium ions and placental thromboplastin (59); the conversion is very slow at pH values acid to 6, but goes rapidly above 6.3 at room temperature (61). The resulting thrombin is much more stable than the prothrombin from which it is formed, although it loses most of its activity within a few days on standing in solution. When dried from the frozen state, it is very stable and retains its full activity for a period of years (61).

The human prothrombin preparations so obtained are still very impure, containing ordinarily 20 or 30 prothrombin units per mg. of protein.\* As

\* The prothrombin unit was originally defined by Seegers, Smith, Warner, and Brinkhous (194) as that amount of prothrombin which, when fully converted to thrombin, will clot 1 cc. of fibrinogen solution in 15 seconds at room temperature. The exact clotting time obtained can vary considerably with the fibrinogen preparation used and with conditions such as pH and salt concentration. In the more recent work in this laboratory, a given preparation of thrombin was taken as a standard, and unknown preparations were assessed with a given fibrinogen preparation, using

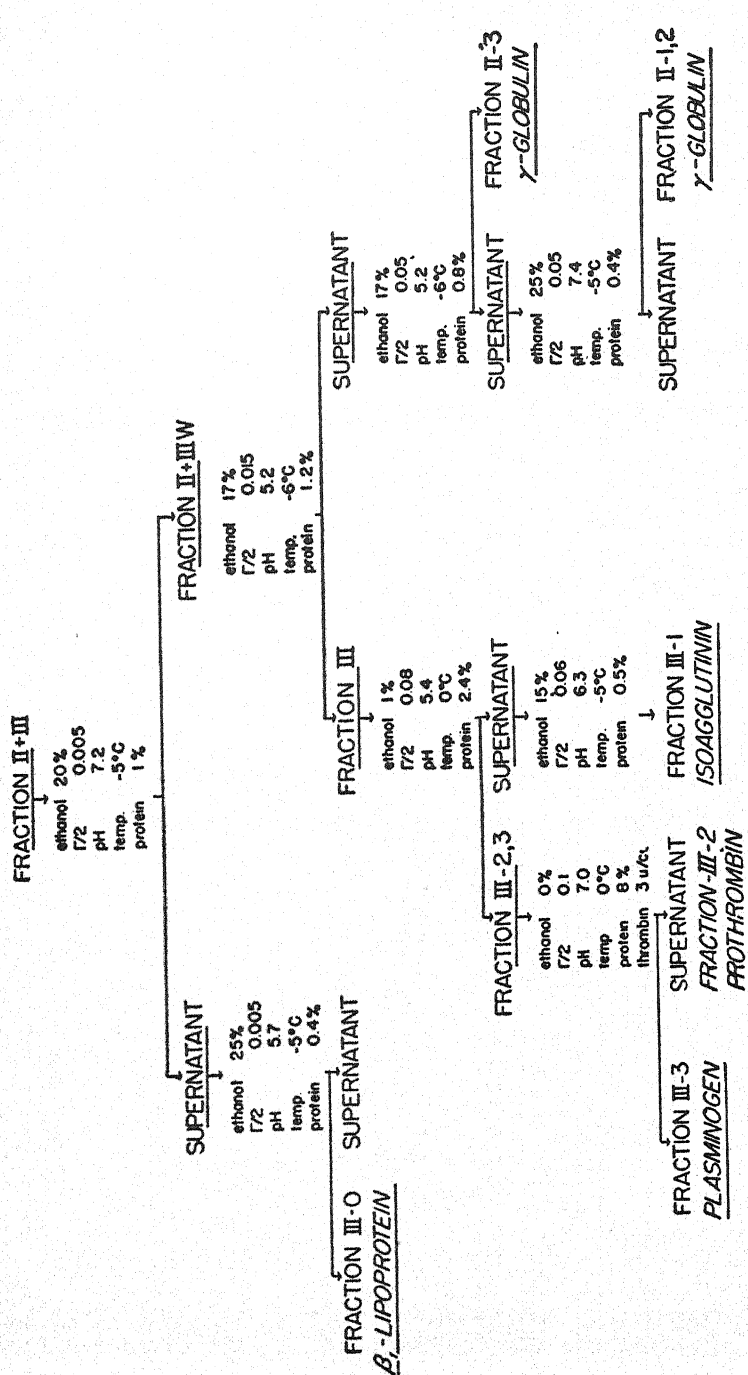


Fig. 9. Steps in the subfractionation of Fraction II+III of human plasma. From L. E. Strong, "Encyclopedia of Chemical Technology."



a result of a long period of research, Seegers and his associates (192, 193) have recently obtained bovine prothrombin in what appears to be pure form. Their method involves an initial precipitation of diluted beef plasma with acid at pH 5.1. This is followed by adsorption on  $Mg(OH)_2$  suspension. The adsorbed prothrombin is eluted by decomposing the  $Mg(OH)_2$  under pressure with  $CO_2$ . The eluate is fractionated with ammonium sulfate in the cold, and finally the prothrombin is separated by isoelectric precipitation. The final product contains approximately 1500 prothrombin units per mg. dry weight. It behaves as a chemical individual by the solubility test. (For the principles involved in the solubility determination, see Northrop (159) and Herriott (97).) It contains 14.5% N, 4.3% carbohydrate (as judged by the orcinol test), and is present in a concentration of approximately 200 mg./l. in bovine plasma.

Highly active thrombin has also been prepared by Milstone (151), by a procedure differing considerably from that of Seegers.

*The Proteolytic Enzyme System of Plasma (Plasmin and Plasminogen).* It has long been known that fibrin clots commonly dissolve after a few hours or days, even in the complete absence of bacterial contamination. It was inferred that this lytic action was due to an enzyme.

In 1903, Delezenne and Pozerski (51) reported that serum became proteolytic when shaken with chloroform, a phenomenon which has been studied in detail by a series of later investigators (see, for instance, Tagnon, 211). This later work indicated that the proteolytic activity induced by chloroform was concentrated in a euglobulin fraction with a minimum solubility near pH 5.2 (212, 112).

In the meantime, the study of fibrinolytic activity had been carried on from what at first appeared to be a totally different point of view. It was found by Tillett and Garner (214, 76) that a very active lytic agent which rapidly dissolved fibrin clots, especially from human plasma, could be extracted from cultures of hemolytic streptococci. The lytic action on human fibrin clots was much more powerful than on clots from most animal bloods. A few years later, Milstone (150) showed that the substance from streptococci did not act on highly purified human fibrinogen; it was necessary to add also a certain euglobulin fraction from human plasma which had a solubility minimum near pH 5.2. The work of Christensen (31) and of Christensen and MacLeod (33) further serial dilutions of both standard and unknown thrombin. The activity of the known preparations was thus directly given by determining the dilution which gave the same clotting time as one unit of the standard. Such standard thrombin preparations are now distributed by the National Institute of Health in order to permit assay of new preparations on a common basis for laboratories throughout the country.

clarified the nature of the reaction. They showed by a series of careful and critical experiments that the streptococcal factor served as an activator for an enzyme precursor present in the euglobulin fraction of normal human plasma; they therefore termed the activator "streptokinase," to indicate its origin and its functional relationship to such other activators as enterokinase. Streptokinase was found to convert the enzyme precursor of plasma into the active enzyme with great rapidity. This enzyme was found to act not only on fibrin and fibrinogen, but on gelatin, casein, and other proteins. It was therefore a true proteolytic enzyme. Its action could be inhibited quite markedly by the crystalline inhibitor which Northrop and Kunitz (159) had prepared and shown to have a high inhibitory action on crystalline trypsin. The form of the inhibition curves was quite different, however, for crystalline trypsin and for the plasma enzyme, indicating their lack of identity. Christensen and MacLeod named the plasma enzyme "plasmin" and its inactive precursor, "plasminogen," and their terminology will be followed here.\* They also prepared activated enzyme from the euglobulin fraction of plasma by chloroform treatment without streptokinase. By every test which they applied the enzyme produced by the action of chloroform behaved like that produced by streptokinase activation.

In the low temperature ethanol fractionation at the Harvard Pilot Plant it was found that plasminogen was highly concentrated in Fraction III-2,3. It was desired to separate it as completely as possible from the prothrombin with which it was closely associated, and which was very similar in its solubility characteristics. In D. A. Richert's method a considerable degree of concentration was achieved by making use of the fact that most of the fibrinogen, not already precipitated in Fraction I, was concentrated in Fraction III-2,3. On the addition of a moderate

\* The terminology in this field has unfortunately been extremely varied. Ferguson (67) has termed the enzyme "serum tryptase" to indicate its general relationship to trypsin as regards pH optimum. However, recent tests by J. S. Fruton (unpublished) on material prepared at the Harvard Pilot Plant by Richert, Miller, and Edsall indicate that the type of peptide linkage split by plasmin is quite different from that split by trypsin. This, in conjunction with the evidence of Christensen, cited above, appears to remove the justification for denoting plasmin by a name suggesting a relationship to trypsin. The name "fibrinolysin" has also been used for the plasma enzyme, as for instance by the present author in a previous article (59). This terminology appears undesirable, since the enzyme is not specific for fibrin, but is a general proteolytic enzyme capable of splitting a certain type of peptide linkage. Moreover, the factor here denoted as streptokinase was referred to in earlier papers as "streptococcal fibrinolysin," the name here denoting an entirely different substance from the plasma enzyme. On the whole, it would appear best to discontinue the use of the name "fibrinolysin" altogether.

amount of thrombin, a fibrin clot was formed which was then centrifuged off, and was found to carry a great deal of plasminogen activity adsorbed on the fibrin clot. On standing in the cold for a day or two, or in a few hours at room temperature, the clot lysed spontaneously, owing to the action of the plasmin which was formed from the plasminogen under these conditions. The resulting fraction was designated as III-3. On holding the preparation at 0° for two or three weeks, a slowly progressive activation occurred without the addition of chloroform, streptokinase, or any other activator. The final concentration of plasmin obtained in this way was about one-third of the maximum obtainable on complete activation with streptokinase. Chloroform treatment of Fraction III-3 did not give any increase in the rate or in the final amount of activation, as compared with the control sample without added activator. This is perhaps explainable by the fact that the lipids present in Fraction II+III were previously separated in Fraction III-0. The function of the chloroform in whole plasma or crude plasma fractions may well be due to its extraction of fat soluble inhibitors which normally prevent the spontaneous conversion of plasminogen to plasmin.\*

Even the most active enzyme preparations yet obtained are still very impure. Further purification will be important, both in making highly active preparations available for possible clinical uses, and in studying the role of the enzyme in the blood coagulation mechanism. Several recent authors, notably Ferguson (66) and Tagnon (211) have stressed its possible importance in the latter connection. Very recently Seegers and Loomis (191a) have reported that plasmin (fibrinolysin) when free of thrombin and prothrombin does not activate prothrombin or clot oxalated plasma or fibrinogen solutions. However, it does *inactivate* prothrombin at a fairly rapid rate, although thrombin is not inactivated by it.

*Isoagglutinin Preparations: Anti-A, Anti-B and Anti-Rh.* The anti-A and anti-B isoagglutinins which react with the specific A and B substances of red cells are concentrated in Fraction III-1, which consists of euglobulins with minimum solubility near pH 6.3 (170, 160, 161). In order to obtain type specific preparations, it is of course necessary that the pooled

\* This conclusion has been strengthened by some very recent work of Christensen (32) who has shown that chloroform apparently acts on serum or plasma by removing the inhibitor of the enzyme which is present in serum. He also found that chloroform treatment of serum caused some destruction of plasminogen, and suggested that the spontaneous activation of plasminogen which occurs following removal of inhibitor might be autocatalytic in nature. He also observed, in agreement with findings from this laboratory, that active plasmin in solution appears to destroy both plasmin and plasminogen activity.



plasma used for processing be taken only from blood of the correct type. In considering the choice of bloods for obtaining active fractions for typing, certain elementary points regarding the nature of blood groups and their frequency in a typical American population must be borne in mind (Table IX).

TABLE IX

Blood Group	Agglutininogen Present in Cells	Agglutinins in Plasma	Per Cent of Population in Group (in U. S. A.)
O	..	anti-A, anti-B	45
A	A	anti-B	41
B	B	anti-A	10
AB	A and B	..	4

Percentage figures from Wiener "Blood Groups and Transfusion"; data of Snyder.

It can be seen from the table that the preparation of anti-B isoagglutinins from donors of type A should be a relatively simple and straightforward problem, granted a suitable method of fractionation. Donors of type A make up more than 40% of the population, and the supplies from this source are ample. To obtain adequate quantities of anti-A isoagglutinins is a more difficult problem. It is of course true that these can be prepared by fractionation of pooled plasmas from donors of type B. However, such donors make up only 10% of the population. This raises a serious difficulty, since equal amounts of anti-A and anti-B agglutinins are required as reagents for typing. Moreover, the anti-A agglutinins from type B plasma are often deficient in their reactions with certain rare sub-types of cells, notably with cells of type  $A_2B$ . The problem of obtaining a large yield of anti-A isoagglutinins was ingeniously solved by Melin (143), by combining bloods of type O and type B as they were obtained at a large blood donor center. Plasma from type O blood contains both anti-A and anti-B agglutinins; and moreover it is more active than type B plasma in its reactivity with cells containing the  $A_2$ -agglutininogen (cells of type  $A_2$  and  $A_2B$ ). The B cells, on mixture with O blood, absorb the anti-B activity almost completely, leaving the anti-A activity in the plasma of the mixed bloods. Cells and plasma are then separated, and the plasma is fractionated in the usual manner, the anti-A agglutinins being concentrated in Fraction III-1. By this means, the plasma available for the preparation of anti-B isoagglutinins from a pool of a typical American population is equal to, or slightly greater than, the amount of anti-B agglutinin available from donors of type A. Furthermore, the

anti-A isoagglutinins, thus prepared from combined O and B blood, are somewhat more active, especially against the rare sub-groups  $A_2$  and  $A_2B$ , than are the isoagglutinins taken from type B donors alone. For a detailed description of the procedure, Melin's article should be consulted; it is to be emphasized, however, that a very careful testing of all the individual bloods used in preparing the O + B pool is essential. A single sample of type A blood, even in a very large pool of O + B blood, is enough to reduce the anti-A activity of the resulting isoagglutinin fraction very markedly indeed.

The assay of isoagglutinin activity proved to raise considerable difficulties, for the techniques employed in different laboratories were so different in detail that the results were not quantitatively comparable, and the red cells used to observe the agglutination reaction inevitably varied somewhat from one individual donor to another. To eliminate these complications, Reference Standard preparations of anti-A and anti-B agglutinins were made up from certain preparations of Fraction III-1, and distributed to all investigators testing new preparations. By assay of the activity of a new preparation against the reference standard, studying both at a series of dilutions, the relative activity of different preparations could be reliably estimated (50).

Just as Fraction III-2 consists of a few per cent of prothrombin, with a large amount of inert protein, so Fraction III-1 consists only of about one per cent of isoagglutinins\*, the rest of the protein in this fraction not being directly involved in the reaction with A and B substances. Thus the properties of pure isoagglutinins cannot be deduced from those of the total protein in Fraction III-1; but this fact does not impair the value of these preparations as blood typing reagents of high titer and avidity.

*Anti-Rh Isoagglutinins.* Although they were discovered only as recently as 1940 by Landsteiner and Wiener, the Rh factors are now recognized as being comparable in importance with the A and B factors, of which they are genetically independent. Anti-Rh isoagglutinins are not found in normal plasma, but are produced in Rh-negative individuals exposed to Rh-positive cells, either by transfusion, or by passage of minute numbers of cells (or breakdown products containing the Rh factor) from an Rh-positive fetus to an Rh-negative mother, across the placental membrane. The grave consequences which may ensue, in the form of the disease *erythroblastosis fetalis* and in other respects, have been discussed in many places.†

\* Personal communication from Dr. E. A. Kabat.

† For a review of the Rh factor, see for instance Boyd (18); also the papers by Levine, and by Wiener and Sonn, in "Annals of the New York Academy of Sciences," vol. 46 (1946).

What concerns us here is the preparation of typing reagents. Anti-Rh agglutinins are present in only a very small fraction of the general population; they must be obtained from sensitized Rh-negative donors, specially selected.\* On fractionation, these agglutinins, like anti-A, and anti-B, are found in Fraction III-1. Fractionation here has proved particularly useful, since most of the whole sera, as collected, are too weak to use as such for typing purposes, but are effective when the agglutinins are concentrated in Fraction III-1.

*The  $\gamma$ -Globulins of Human Plasma.* The  $\gamma$ -globulin fractions, II-1,2 and III-3, obtained by the procedures outlined on p. 447-8, are highly pure by the criterion of electrophoretic mobility. Preparations containing 98%  $\gamma$ -globulin, or better, are readily obtained. However Oncley's ultracentrifugal studies (162) (see also Williams *et al.*, 217) have shown that only 75-85% of the protein in this fraction sediments with the value  $s_{20}=7$  S; the remainder consists of one or more faster moving components, with  $s_{20}=10$  S approximately. Furthermore this fraction has been shown by repeated tests, both immunological and clinical, to contain a large number of antibodies. Those determined by laboratory procedures include antibodies to diphtheria, influenza, mumps, whooping cough, scarlet fever, poliomyelitis, lymphocytic choriomeningitis, certain streptococci, vaccinia and typhoid.† The evidence, derived from studies by many workers, is summarized by Enders (62) in a comprehensive paper.

Moreover extensive clinical trials have shown the great utility of such preparations in producing temporary passive immunization to measles (163, 203) and to infectious hepatitis (204, 77, 92), and have thereby demonstrated that antibodies to both these diseases are present in the  $\gamma$ -globulin fraction of human blood. Unquestionably many other antibodies are present also in this fraction, and have not yet been detected simply because they have not been tested for.

\* The presence of "blocking" antibodies in many such anti-Rh sera (53) further complicates the problem of observing the agglutination reactions. Agglutination of Rh-positive cells by such sera is greatly improved by the addition of whole plasma or various plasma fractions. Serum albumin, because of its ready availability in large quantities today, has been found particularly useful as a reagent to aid in Rh typing determinations (25).

† The typhoid H agglutinin is found in Fractions II-1, 2 and II-3; but the typhoid O agglutinin is found predominantly in Fraction III-1.

‡ All the earlier studies were made with Fraction II preparations essentially equivalent to what is now denoted as II-1,2. Fraction II-3 represents additional  $\gamma$ -globulin, not recovered in the earlier procedures. Its content of nearly all antibodies (including measles antibody) is virtually identical with that of II-1,2; at present (July, 1946) no opportunity has arisen to test its effectiveness in infectious

The  $\gamma$ -globulin of Fraction II is readily prepared in aqueous solutions containing 16% or more of protein. The  $\gamma$ -globulin and antibody content of such solutions, per unit volume, are approximately 25 times as great as in the plasma used as starting material. By fractionating plasma derived from donors convalescent from a particular disease, the specific antibody content of the original plasma is much increased; and the concentrated Fraction II from such plasmas may become a powerful agent in the treatment of certain diseases. Studies of this sort, using donors convalescent from mumps, from scarlet fever and from other diseases have already begun.

Studies have been made in the Wisconsin laboratories of the enzyme digestion of human  $\gamma$ -globulin. Bridgman (22a) found that pepsin digestion at pH 3.5 led chiefly to the production of molecules of approximately half the original size,  $s_{20}$  falling from 7 to 6 S. The optimum conditions for half-molecule production involved digestion for three days, in the cold, with at least 0.05 hemoglobin units of pepsin per g. globulin. Further digestion led to the production of still smaller molecules and eventually of dialyzable fragments. On immunological assay of the preparations of half-molecules, it was found that most of the antibody activities were present in undiminished potency, although the typhoid "O" agglutinin was destroyed.

Digestion by papain or bromelin (Petermann, 167a), on the other hand, led to the formation of quarter-molecules ( $s_{20}=4.1$  S) with only a small increase in non-protein nitrogen. Electrophoretically the digestion products were very inhomogeneous, giving a series of different components, in sharp contrast to the pepsin digestion products, which were homogeneous in this respect and practically identical in mobility with the undigested globulin. The quarter-molecules still possessed some antibody activity, although considerably less, with respect to most of the antibodies tested, than was present in the original globulin.

Deutsch, Petermann and Williams (52b) developed a combined pepsin digestion and fractionation system to recover half-size  $\gamma$ -globulin antibodies from an initial fraction containing  $\beta$  and  $\gamma$ -globulins.\* The half-size antibodies give less viscous solutions, and diffuse more rapidly, than the original molecules, and these modified properties may be valuable for clinical use.

Deutsch, Gosting, Alberty and Williams (52a) found that the less soluble fraction of the  $\gamma$ -globulin was best separated from  $\beta$ -globulin at pH 5.1 and at low ionic strength (near 0.01). Under these conditions, at 17 per hepatitis. It seems probable that for clinical use, the two subfractions can be combined into a total Fraction II ( $\gamma$ -globulin).

\* This fraction was denoted as III-1 in an earlier fractionation method 3 C (see Oncley *et al.*, 161). The  $\gamma$ -globulin present in this fraction was later separated in Fraction II-3 in method 9.

cent ethanol and  $-6^{\circ}$ , the  $\gamma$ -globulin remains in solution while the  $\beta$ -globulin is precipitated. This observation represented one of the important steps leading to the separation of Fraction II-3 in method 9.

Separation of a globulin with electrophoretic mobility slightly greater than that of ordinary  $\gamma$ -globulin has been reported by Deutsch, Alberty and Gosting (51a). They denote this new fraction as  $\gamma_1$ -globulin (other authors have denoted it as  $\beta_2$ ) and report in detail methods for its separation from the bulk of the  $\gamma$ -globulin fraction (which they denote as  $\gamma_2$ -globulin).

#### 4. *Subfractionation of Fraction IV-4; Serum Esterase and the Iron-Binding Protein of Plasma*

Fraction IV-4 consists of nearly lipid-free  $\alpha$ - and  $\beta$ -globulins, with some albumin. It contains at least two components of major biochemical interest: namely serum esterase and a specific iron-binding globulin.\* The esterase, although it readily hydrolyzes acetylcholine, is not specifically a choline esterase, like the cholinesterase of red cells or brain; it also hydrolyzes tributyrin and other simple fatty acid esters.† The esterase from horse serum has been very highly purified by Strelitz (205) who, using ammonium sulfate fractionation, obtained preparations 5000 times as active as the original serum, in enzyme activity per mg. protein. In the subfractionation of IV-4 from human plasma, the esterase activity is strongly concentrated in a fraction known as IV-6; this consists mostly of  $\alpha_2$ -globulin, but the esterase has not yet been sufficiently purified to identify it definitely with any electrophoretic component.

It was recognized many years ago by Barkan (10) that serum contains a small amount of iron, which is readily split off on acidification. Later it was found that this iron-binding power was associated with the globulin fraction of blood, not with the albumin (Barkan and Schales, 11). The iron-binding capacity of human plasma is concentrated in a subfraction of IV-4, now denoted as IV-7 (Schade and Caroline). This fraction consists chiefly of a  $\beta_1$ -globulin, which is however entirely different in its properties from the  $\beta_1$ -globulin which is the chief electrophoretic constituent of Fraction III-2, or the  $\beta_1$ -lipoprotein of Fraction III-0 (X-

\*Hypertensinogen is present in high concentration in the supernatant, after precipitation of Fraction IV-1; however it is largely inactivated at the high ethanol concentration employed in precipitating Fraction IV-4. Assay of hypertensinogen has been carried out by Dr. Lewis Dexter of the Peter Bent Brigham Hospital.

† Concerning the specificity of serum esterase see Mendel and Rudney (148), Mendel, Mundell and Rudney (147); concerning the different action of various inhibitors on the esterases from serum, red cell, and brain, see Zeller and Bissegger (222).

protein). The  $\beta_1$  component of Fraction IV-7 is a small molecule, with a molecular weight near 90,000. It is as yet not certain, however, that the iron-binding globulin is to be identified with a  $\beta_1$ -globulin. In any case this iron-binding function of plasma depends on the specific action of a particular globulin fraction; free iron ( $\text{Fe}^{++}$  or  $\text{Fe}^{+++}$ ) added to serum is rapidly bound by this particular component; excess iron above the binding capacity is rapidly eliminated from the circulation and is toxic. Holmberg and Laurell (106) report the iron-binding capacity of normal serum as in the neighborhood of 3 mg. Fe per liter. The probable physiological significance of this protein-bound iron, for the transport of iron in the body, was early pointed out by Barkan (10) and all our present knowledge would appear to confirm his views.

#### 5. *Crystallization of Serum Albumin from Ethanol-Water*

Both human and bovine serum albumins have been crystallized from ethanol-water mixtures at  $-5^\circ$  (Cohn and Hughes, 40). Needle-shaped crystals of bovine albumin were obtained from 15% ethanol at pH 5.1 and ionic strength 0.02; crystals of more compact form were obtained in 40% ethanol at ionic strength 0.4 and pH 5.5. Human albumin was first crystallized from 40% ethanol, from albumin solution that had been concentrated in a vacuum still, with decanol added to prevent foaming. Albumin precipitated as Fraction V, however, without decanol could not be crystallized under the same conditions unless, as Hughes discovered in 1942, decanol (about 0.1%) was added to the system. Hughes proceeded to show that other aliphatic alcohols containing six or more carbon atoms were also effective in promoting crystallization, as were toluene and benzene. These added substances appear to be bound to the albumin and become a part of the crystals. This whole effect is intimately related to the specific tendency of serum albumin to bind many organic molecules and ions (see Part VII). Such experiences indicate the importance of taking into account even components present in minute amounts, when solubility equilibria are formulated in systems containing proteins.

#### 6. *The Lipoproteins of Plasma*

The presence of lipids and steroids in close association with certain plasma protein fractions has often been observed. An excellent brief account of some of this earlier work has already been given by Chargaff (28) in an earlier volume of this series. Macheboeuf (134) in 1929 obtained a water-soluble (or salt-soluble) lipoprotein from horse serum by precipitating most of the globulin with half-saturated  $(\text{NH}_4)_2\text{SO}_4$ , and acidifying the filtrate to pH 3.8. The precipitate so separated was

redissolved at pH 7, and repeatedly reprecipitated at pH 3.8. The final product contained 17.9% cholesterol esters and 22.7% phospholipids, and was soluble in water to a concentration of 50 g./l. (See also 135.)

Blix, Tiselius and Svensson (16) studied the lipid content of electrophoretically separated albumin and  $\alpha$ -,  $\beta$ -, and  $\gamma$ -globulins from human serum. The lipid was associated chiefly with the  $\alpha$ - and  $\beta$ -globulin fractions.\*

Adair and Adair (1) obtained a globulin fraction of low density from human serum, by precipitation between 0.5 and 0.6 saturation with  $(\text{NH}_4)_2\text{SO}_4$ . They found 8.5% phospholipid, 16.5% cholesterol, and 20.4% of fatty acids in the reprecipitated protein. The molecular weight from osmotic pressure was 370,000.

In this laboratory, two sharply distinct lipoproteins, both euglobulins and both highly soluble in aqueous saline solutions, have been obtained by the low temperature—low salt—ethanol fractionation: an  $\alpha_1$ -lipoprotein from Fraction IV-1, and a  $\beta_1$ -lipoprotein from Fraction III-0. The former has a molecular weight of the order of 200,000, and the latter of the order of 1,000,000 (Oncley, Scatchard and Brown, 162). Some of the properties of these lipoproteins are given in Table X, together with

TABLE X

*Lipoproteins Isolated from Plasma*

Investigators	Principal Electrophoretic Component	Sedimentation Constant * <sub>20, w</sub>	Per Cent (g. per 100 g. Dry Protein)		
			Nitrogen	Cholesterol	Total Lipid
Macheboeuf, 1928	( $\alpha_1$ )	4.1	8.0	18.	50
Blix, Tiselius and Svensson, 1941	$\alpha$	...	...	4.4	...
Blix, Tiselius and Svensson, 1941	$\beta$	...	...	9.	...
Adair and Adair, 1943	...	...	6.4	16.5	46
Pedersen, 1945	...	6.	...	...	...
Cohn, Blanchard and Strong, 1946	$\alpha_1$	5.	10.	16.	35
Oncley, Melin and Gross, 1946	$\beta_1$	6.	4.	35.	75

those reported by other investigators. It should be noted that the  $\beta_1$ -lipoprotein (Pedersen's X-protein) although containing 75% lipid, is

\* Blix, Tiselius, and Svensson reported also appreciable amounts of cholesterol and phospholipid in their albumin and  $\alpha$ -globulin fractions. This probably indicates that these fractions were impure, since Cohn, Strong *et al.* (45) report <0.06% cholesterol in chemically purified  $\gamma$ -globulin, and <0.04% in albumin.



soluble to the extent of 10% or more in dilute aqueous salt solutions. It can be repeatedly reprecipitated at low ionic strength, and redissolved by addition of salt, without denaturation. Both it and the  $\alpha_1$ -lipoprotein, however, are denatured, with release of lipid, on exposure to high alcohol concentrations, or on freezing to temperatures below  $-25^\circ$ , (see McFarlane, 133).

The carotenoids of plasma are found, for the most part, in the  $\beta_1$ -lipoprotein,\* although some carotenoid is also present in the  $\alpha_1$ -lipoprotein. The latter contains most of the bile pigments of plasma. The steroids of plasma, including the estrogens, are found in the  $\beta_1$ -lipoprotein (177a). This protein is also remarkable in being apparently characteristic only of human plasma, in all the species yet investigated. Pedersen (166) says that it was not detected in any other plasma during his ultracentrifugal studies, and fractionation studies in this laboratory on several animal plasmas are entirely in accord with his statement.

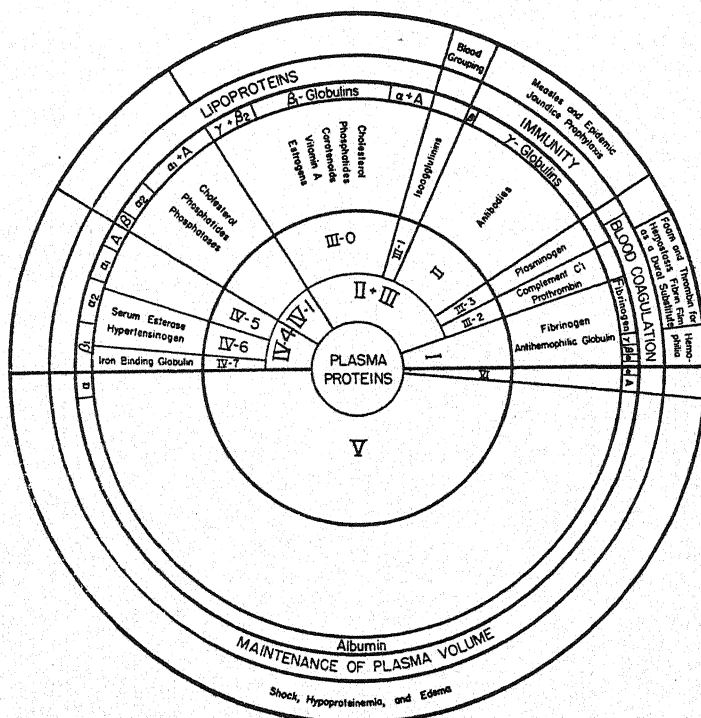


Fig. 10. Plasma proteins. Their natural functions and clinical uses and separation into fractions.

\* Unpublished work of J. W. Mehl in the Harvard Plasma Fractionation Laboratory.



TABLE XI  
*Protein Components of Normal Human Plasma Characterized by Physical Chemical Methods*  
(Oncley, Scatchard and Brown, 1962).

Electrophoretic Component	Fraction	Approximate Amount in Plasma (g./l.)	Sedimentation Constant $s_{20,w}$	Specific Volume $V$	Intrinsic Viscosity $H_0 \times 10^3$	Frictional Ratio $f/f_0$	Molecular Weight $M$	Approximate Dimensions (Angstroms)	
								Length	Diameter
Albumin	V	32	4.6	0.733	4.2	1.28	69,000	150	38
$\alpha_1$ -globulin	IV-1	2*	5.0	.841	6.6	1.38	200,000	300	50
$\alpha_2$ -globulin	IV-6	1	9.	.693	9.2	1.58	(300,000)	...	...
$\beta$ -globulin	IV-7	2	5.5	.725	5.5	1.37	90,000	190	37
$\beta$ -globulin	III-0, III-2	2	7.	.74	...	...	(150,000)	...	...
$\beta$ -globulin	III-0	1	20.	.74	...	...	500,000-	...	...
$\beta$ -globulin	III-0	2*	2.9*	.950	4.1	1.7 <sup>a</sup>	1,000,000	...	...
$\beta$ -globulin	III-1	2	7.	...	...	...	1,300,000	185	185
$\gamma$ -globulin	II	5	7.2	.739	6.	1.38	(150,000)	...	...
$\gamma$ -globulin	II	1	10.	.739	...	...	156,000	235	44
Fibrinogen	I-2	2	9.	...	25.	1.98	(300,000)	...	...
							400,000	700	38

\* These two globulins are lipoproteins containing 35% lipid for the  $\alpha_1$ -globulin and 75% lipid for the  $\beta$ -globulin. The other components contain little or no lipid.  
<sup>a</sup> This is the sedimentation constant obtained in 0.5 M sodium chloride solution when corrected in the usual manner. The  $f/f_0$  value given here is the value for sedimentation, correcting for the partial specific volume of the lyophilized protein (0.97), using the equation of Kraemer (equation 124, p. 65, reference 208). A solvation of 0.6 g. of water per g. of protein was used for this calculation, and the molecule was assumed spherical.

The distribution of human plasma proteins into fractions and sub-fractions is diagrammatically represented in Fig. 10 (for an earlier version, see Cohn 37, 38, or Oneley *et al.*, 160).

### 7. Ether Fractionation of Human Plasma

In England, during the war, precipitation with ether at low temperature has been employed as a method of separation of fibrinogen and prothrombin from plasma (Kekwick, Mackay and Record, 114). Ether is soluble in plasma, at 0° to -0.5°C., to 11 vols. per 100 cc., and on addition of ether to this concentration 90% of the total plasma fibrinogen is precipitated. The precipitate is only about 40% fibrinogen, but can be further purified by reprecipitation with ether. The plasma supernatant, after precipitation of fibrinogen, is adjusted to pH 5.3-5.4 with citric acid. A yellow precipitate is formed, containing prothrombin, which is then converted to thrombin. Thus the starting materials for fibrin foam, fibrin film, and other products related to fibrinogen, are obtained. Ether is not sufficiently soluble in plasma to permit further fractionation; instead, the residual liquid, after prothrombin removal, is frozen with ether below -25° (McFarlane, 133), thus largely breaking the bonds between protein and lipid in the lipoproteins, the lipids are extracted, and the remaining clear fluid can be used for clinical injection in shock and other conditions. Presumably, if used on a large scale, this method must be employed with special precautions, on account of the inflammability of ether.

### 8. Size and Shape of Molecules in Plasma Fractions

Studies of sedimentation constant, viscosity, osmotic pressure, and partial specific volume of plasma fractions have permitted estimation of molecular weights and axial ratios for many of the components involved (Oneley, Scatchard and Brown, 162). The results are summarized in Table XI and the ultracentrifugal data are also presented in Fig. 11. The height of each bar in the figure indicates the weight of that fraction obtained during fractionation. For Fraction V several bars are placed side by side, and the heights should be added together, this fraction representing 48% of human plasma protein by weight. In Fig. 12 a similar diagram is shown for the electrophoretic components from the various fractions. It is immediately apparent that there is no simple correspondence between the ultracentrifugal and the electrophoretic components. At least four distinct  $\beta_1$ -globulins have been identified, and it seems almost certain that other quite distinct  $\alpha$ - and  $\beta$ -globulin components are still to be identified. The shapes of the molecules vary from that of the  $\beta_1$ -lipoprotein, which is very large and practically spherical,

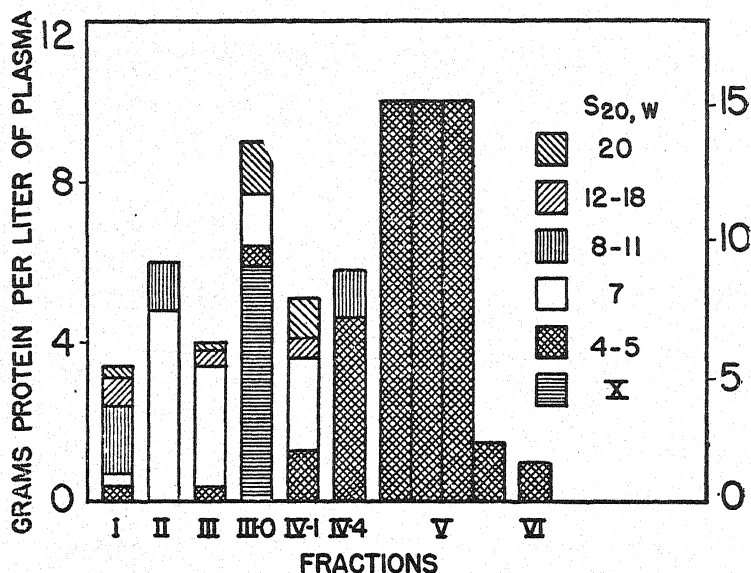


Fig. 11. Distribution of ultracentrifugal components in fractions of human plasma. Three bars, and a portion of a fourth, are necessary to represent the composition of Fraction V (albumin), which makes up 48 per cent by weight of the total human plasma protein. From Oncley, Scatchard and Brown (162).

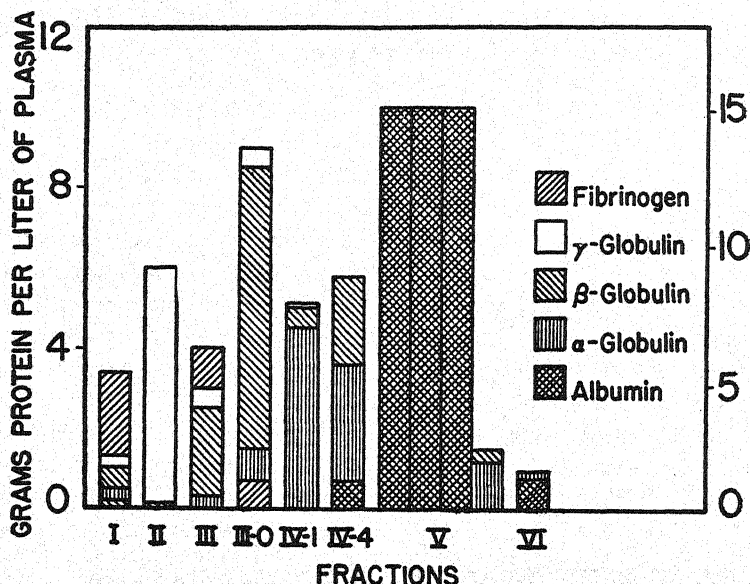


Fig. 12. Distribution of electrophoretic components in fractions of human plasma, arranged on the same plan as Fig. 11. From Oncley, Scatchard and Brown (162).

to the long thin molecule fibrinogen, 700 Å long and about one-twentieth as wide.

### 9. *Amino Acid Analysis of Plasma Fractions*

Accurate data on the amino acid analysis of plasma fractions are now available from the work of Brand and his co-workers (21, 22), who have employed chiefly colorimetric and microbiological procedures, while Shemin (195) has determined the content of several amino acids in human and bovine serum albumin by the isotope dilution method. When Brand's determinations were checked against those obtained by isotope dilution, excellent agreement was obtained. The data, given as per cent amino acid in the hydrolyzate from 100 g. protein, are given in Table XII; and for human and bovine serum albumin, and human  $\gamma$ -globulin, they are given as residues per mole in Table XIII. It will be noted that human and bovine serum albumin, although strikingly similar in composition in most respects, differ significantly in their content of methionine, tryptophan, isoleucine and certain other amino acids. The very high content of hydroxyamino acids in  $\gamma$ -globulin, and the still high but somewhat lower content of these acids in fibrinogen, are notable. Nutritional studies with rats (27, 94) have shown that fibrinogen is the most complete of these proteins, as a component of the diet.  $\gamma$ -Globulin is nearly as good, while human albumin is deficient in tryptophan and isoleucine. When supplemented with these two amino acids, albumin gives good growth (93).

## VII. REVERSIBLE COMBINATION OF SERUM ALBUMIN AND OTHER PLASMA PROTEINS WITH SMALL MOLECULES OR IONS; FACTORS AFFECTING STABILITY TO HEAT

Plasma and serum are notable for their capacity to hold in stable aqueous solution many substances whose solubility in pure water or salt solutions is extremely low. The remarkable capacity of certain globulin fractions—notably those from Fractions III-0 and IV-1, as separated by the low temperature-ethanol technique—to bind cholesterol, phosphatides, and other types of lipid material, in stable, water-soluble combination has already been described in the preceding section. Serum albumin, however, displays equally remarkable affinities for a somewhat different and extremely diverse group of substances, including many types of acid and basic dyes, the ions of many organic acids containing hydrocarbon residues, certain quinone derivatives, and other substances.

In 1922 it was discovered independently by de Haan (86) and by Marshall and Vickers (141) that certain dyes are strongly bound to the plasma proteins. Marshall and Vickers were particularly concerned with

TABLE XII  
*Amino Acid Composition of Human Plasma Proteins*  
 g. per 100 g. Protein

Constituent	Albumin	$\gamma$	$\beta$	$\alpha$	Fibrinogen
Total N	15.95	16.03	15.24	...	16.9
Total S	1.96	1.02	1.32	...	1.26
Free $\alpha$ -amino N	0.18	0.11	...	...	...
Amide N	0.88	1.11	...	...	...
Glycine	1.6	4.2	5.6	3.1	5.6
Alanine	...	...	...	...	...
Valine	7.7	9.7	7.0	5.2	4.4
Leucine	11.0	9.3	7.9	14.2	7.1
Isoleucine	1.7	2.7	5.0	1.7	4.8
Proline	5.1	8.1	7.1	4.7	5.7
Phenylalanine	7.8	4.6	4.7	4.6	4.2
Cysteine	0.7	0.7	{ 3.5 }	{ 1.5 }	0.4
Half-Cystine	5.6	2.4			2.3
Methionine	1.3	1.1	1.7	1.4	2.5
Tryptophan	(0.2)	2.9	2.0	1.9	3.3
Arginine	6.2	4.8	6.8	7.7	7.9
Histidine	3.5	2.5	2.8	2.8	2.8
Lysine	12.3	8.1	6.6	8.9	8.3
Aspartic Acid	10.4	8.8	9.8	9.0	13.6
Glutamic Acid	17.4	11.8	14.5	21.6	14.3
Serine	3.7	11.4	7.1	5.0	9.2
Threonine	5.0	8.4	6.1	4.9	6.6
Tyrosine	4.7	6.8	6.0	4.5	5.8
Totals	105.9	108.3	104.2	102.7	108.8

Albumin (V): No. 42.

$\gamma$ -Globulin (II-1): No. 36.

$\beta$ -Globulin (III-22 $\beta$ ): No. L-421.

Alb. = 1%,  $\gamma$  = 1%,  $\alpha$  = 7%,  $\beta$  = 82%,  $\beta_2$  = 9%.

Ash = 8.13%, values on ash-free basis.

$\alpha$ -Globulin (IV-1): No. Run 146.

$\alpha$  = 94%,  $\beta$  = 6%, lipids = 35.2%.

Values on lipid-free basis.

Fibrinogen (I): No. 81 R.I. 87% clottable.

Data from Brand, Kassell and Saidel (22); Brand (21); Shemin (195).

phenol red, and Grollman (84), two years later, made a more detailed study of the binding of this substance by serum proteins, gelatin and egg albumin, from which he concluded that the binding power of serum globulin was very small compared with that of albumin. There was a

marked effect of pH on the amount bound. The maximum binding occurred in fairly acid solution, and the binding was very weak at pH values alkaline to 8. Rawlins and Schmidt (174) made similar studies concerning the combination of gelatin with Biebrich's scarlet and tropeolin 0, and

TABLE XIII

*Amino Acid Content of Human and Bovine Serum Albumin  
and Human  $\gamma$ -Globulin Expressed as Residues Per Molecule of Protein*

	Human Serum Albumin	Bovine Serum Albumin	Human Serum $\gamma$ -Globulin
Assumed mol. wt. $\times 10^{-3}$	70	70	156
Average Residue wt.	119.4	118.7	113.6
Residues per mole (approx.)	586	590	1373
Total N atoms/mole	797	803	1785
Free $\alpha$ -amino N	9	10	12
Amide $\text{NH}_2$	44	43	124
Arginine	25	25	43
Histidine	16	17	25
Lysine	59	60	86
Total cationic groups*	109	112	166
Aspartic Acid	52	54	103
Glutamic Acid	81	81	126
Free carboxyl groups**	98	102	117
Total Protein S	42	42	50
Cysteine	4	6	9
Half Cystine	32	32	30
Methionine	6	4	11
Tryptophan	(0.6)	2	22
Serine	25	30	169
Threonine	30	38	110
Tyrosine	18	21	58
Glycine	15	18	87
Valine	46	39	129
Leucine	64	73	111
Isoleucine	9	15	32
Phenylalanine	33	26	44
Proline	31	34	110
Total Residues Accounted For	551	575	1305

\* Total cationic groups are taken as the sum of the arginine, histidine, lysine, and  $\alpha$ -amino residues.

\*\* Free carboxyl groups are taken as the sum of the total aspartic and glutamic acid plus free  $\alpha$ -carboxyl (assumed equal to free  $\alpha$ -amino) minus the amide nitrogen groups.

Data for the alanine content of these proteins are not yet available.

Data from Brand, Kassell and Saidel (22) and from Brand (21).

concluded that specific chemical interactions between the dye and certain groups of the protein molecule were involved.

Far-reaching advances in our knowledge of these phenomena were achieved in the work of Bennhold (12, 13). He employed the ingenious and simple technique of observing the diffusion of various dyes from an aqueous layer downwards into a gelatin gel, in some cases mixing serum proteins with the aqueous dye solution, while in the controls the serum was absent. Under these circumstances he found, for instance, with the dye naphthol yellow S, that after four days the free dye in water had penetrated 36 mm. into the gelatin layer, while in the tube in which serum had been added, it penetrated only 8 mm. Furthermore, analyses of successive layers in the second gelatin gel showed that the serum protein had penetrated exactly as far as the dye, but no further—in other words, the dye and the protein had moved together and were apparently firmly bound together. Studies on another type of dye, congo red, gave essentially identical results in the presence of serum. Congo red, however, in aqueous solution is present in the form of large colloidal aggregates which scarcely diffuse into the gelatin gel at all. In this case, the addition of serum protein actually favors the diffusion of the dye by causing the disaggregation of the colloidal particles of congo red, and the attachment of the individual molecules to those of the serum protein. Analogous findings were obtained by Bennhold with a long series of dyes of varied structure, and the effects were essentially similar for both acid and basic dyes.

While Bennhold's first studies were made on whole serum, he later proceeded to study serum fractions, and showed that the characteristic dye binding in most cases was due almost entirely to the albumin fraction. These conclusions were further extended by a long series of studies on the cataphoresis of serum proteins and protein fractions, using the type of apparatus developed by Michaelis. The attachment of these various dyes to the proteins was clearly revealed, and the color of the dye-protein complex permitted the observation of a well-defined boundary. Definite limits to the binding capacity of serum albumin for dye were observed in these studies. Above a certain critical concentration free dye was recognizable in solution and moved in the electric field with a velocity different from that of the albumin-dye complex. Bennhold also showed that bilirubin, when added to serum, was quantitatively bound to the albumin, up to a certain limited binding power.

He also made some interesting observations on the attachment of cholesterol to the serum globulins, and used colloidal sudan red to stain the globulin-cholesterol complex in order to permit easy observation of

the boundary during the cataphoresis experiments. He concluded that the serum albumins normally bind specifically no cholesterol, while the globulins, at least certain globulins, bind it readily. These points need not be further discussed, since they have been so amply confirmed by the later data already presented in Part VI. Bennhold drew far-reaching inferences from these observations, concluding that one of the major functions of the serum proteins is to serve as a vehicle for transport of smaller molecules and ions. While most of his observations were made with synthetic chemicals, having presumably no normal biological function, it was reasonable to assume that similar transport mechanisms operated for many substances normally present in the living organism. While Bennhold gave no definitive proof of these conceptions, the weight of a large amount of evidence since obtained has substantiated and even extended them.

In a later report, Bennhold (13) stated that atabrin, when added to plasma, is almost entirely bound to albumin; however, its affinity for red cells is so great that in whole blood it is nearly all found in the cellular phase. Albumin, however, may well act as a transporting agent in the transfer of atabrin between red cells and tissue cells. The studies of Davis (47) have shown that sulfanilamide and other sulfa drugs are bound largely to albumin in plasma, and do not appear to enter into combination with the globulins at all.

In the course of the plasma protein fractionation program, while it was being vigorously prosecuted during the war, an entirely different type of problem led to studies which revealed similar underlying mechanisms. In the preparation of concentrated solutions of serum albumin for the armed forces, it was of vital importance that the albumin solution should be stable, even at relatively high temperatures, over long periods of time. On account of the need for albumin in tropical and desert warfare, exposure of the material to high temperatures was to be expected. Therefore a thermal stability test was imposed on these solutions before they were released for clinical use. Samples were observed for a period of at least 12 days at a temperature of 50°C., and the light scattering before and after was measured in a nephelometer. Any significant rise in the nephelometric reading was considered sufficient ground for rejection of the preparation. It was soon observed that stability to heat, as judged by this test, depended upon the concentration of sodium chloride present, and increase from 0.15 to 0.3 *M* produced a very marked increase in heat stability (184). It was then observed in the laboratory of J. M. Luck at Stanford that sodium acetate enhances thermal stability considerably more than an equivalent molar



concentration of sodium chloride. Further studies with higher members of the fatty acid series revealed that the stabilizing effect increased progressively with the length of the hydrocarbon chain from acetate to caprylate (8, 7). To study this phenomenon, the albumin solution was observed in a thin walled capillary tube, and was heated to a constant and comparatively high temperature until a sharply discernible cloud formed in the solution. It proved convenient to choose as a standard of measurement the temperature which gave a cloud point in 30 seconds for a given solution. The results for chloride and a series of fatty acid anions at various concentrations (20) are shown in Fig. 13. The 30 second

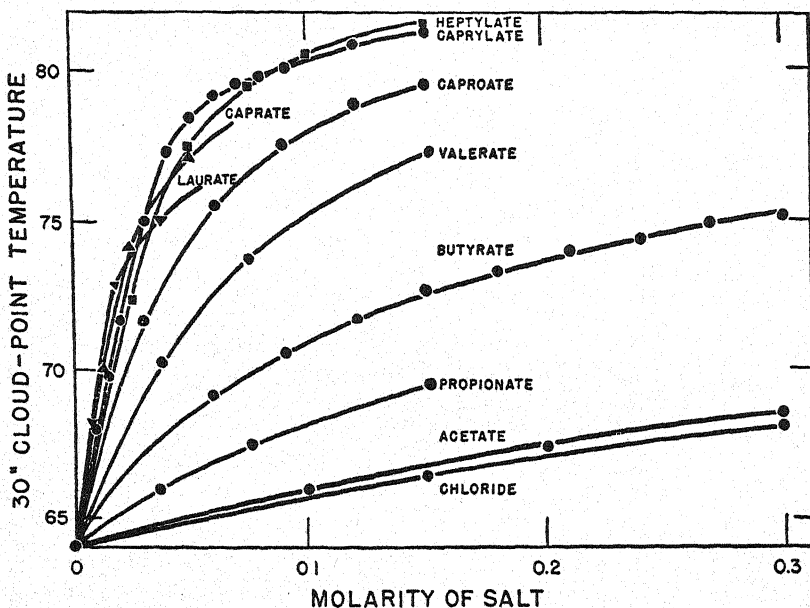


Fig. 13. The stabilization of serum albumin to heat by fatty acid salts. From Boyer, Lum, Ballou, Luck and Rice (20).

cloud point temperatures shown in this figure vary over a range of less than  $20^{\circ}\text{C}.$ ; but on account of the great temperature coefficient of the heat denaturation of albumin, these results imply a prodigious increase in the possible length of heating of an albumin solution at constant temperature, when a dilute solution of a salt such as sodium caprylate is added.

A systematic study of a series of related compounds showed that the essential type of structure necessary for the stabilization of albumin consisted of an anionic group to which was attached a fairly large non-polar residue, either aliphatic or aromatic. "Twenty-three compounds,

including amino acids, peptides, proteins, sugars, alcohols, amides, esters, and amines with non-polar groups, did not possess the marked stabilizing properties of the non-polar anions" (20, p. 187). The presence of additional hydroxyl or carboxyl groups in the non-polar residue decreases the stabilizing effect of any given anion. Thus, mandelate at a given molar concentration has much less stabilizing effect than phenylacetate, and phthalate less than benzoate. The stabilizing effect of alkyl sulfonates is of the same order of magnitude as that of the corresponding fatty acid anions, although somewhat less in proportion to size. Cations with attached non-polar groups diminished the stability of albumin.

The fact that the stabilizing anions are actually bound to the protein was shown on the one hand by electrophoretic measurements, which showed a marked increase in mobility in the presence of butyrate, caproate, heptate, caprylate, and phosphate ions; and on the other hand by ultrafiltration studies.

Electrophoretic studies indicating combination between detergent anions and proteins have been reported by Lundgren, Elam, and O'Connell (131), who studied the system composed of albumin and alkylbenzenesulfonates, and by Putnam and Neurath (172, 173) who employed horse serum albumin and sodium dodecyl sulfate. The latter authors observed three electrophoretic components in phosphate buffer at pH 6.8, ionic strength 0.2. These were identified by their respective mobilities of 4.9, 7.8, and  $9.8 \times 10^{-5}$ . The first was assumed to be free albumin (A). The second component, which they denoted  $AD_n$ , was believed to contain 55 moles of D per mole of A, corresponding approximately to one-half the maximum acid binding capacity of A. The third component,  $AD_{2n}$ , was believed to contain 110 moles D per mole of A. The formation of the latter complex appeared from viscosity measurements (157) to involve unfolding of the albumin molecule, while that of the complex  $AD_n$  did not.

The formation of such sharply distinct compounds without intermediate components is surprising, and the system merits further study. However, all these studies demonstrate beyond doubt that complex formation does occur between negatively charged protein molecules and smaller negatively charged ions. The stoichiometric relations involved suggest that the positively charged residues of the dibasic acids in the albumin molecules must play a fundamental part in the compound formation. Obviously, however, van der Waals forces between non-polar residues, in the attached ion and in the protein molecule, must also play a major part in stabilizing the complex.

The influence of fatty acid anions on the denaturation of human and bovine albumin by urea or guanidine hydrochloride was studied by

Boyer, Ballou, and Luck (19). Low concentrations of fatty acid anions prevented the viscosity increases which otherwise resulted when the albumin was dissolved in 6 *M* urea. The effect of the fatty acid anions increased with the chain length. Caprylate also prevented the viscosity increase which otherwise occurred when the albumin was dissolved in 2.5 *M* guanidine hydrochloride, but it had no stabilizing effect in the presence of 6 *M* guanidine hydrochloride. Serum  $\gamma$ -globulin was not protected from urea denaturation by the addition of caprylate.

The study of the stabilizing effect of various anions was simultaneously being pursued at the Harvard Laboratories, with the aim of finding stabilizers for albumin of particular value for the armed forces. Scatchard, Strong, Hughes, Ashworth, and Sparrow (186) carried out independent studies on some of the same compounds observed by Luck and his co-workers, and also made an intensive study of the sodium salt of the acetyl derivatives of certain amino acids, notably phenylalanine, isoleucine, and tryptophan.\* The use of the latter two amino acid derivatives was proposed by Dr. L. E. Strong on the basis of analytical (22) and nutritional studies (93, 94), which suggested that serum albumin was deficient in both of these amino acids. The sodium salt of acetyltryptophan, in particular, proved an extremely effective stabilizer.

In the presence of 0.04 molal acetyltryptophanate, it was possible to heat concentrated serum albumin solutions for 10 hours at 60° without causing an undue rise in the turbidity of the solution, as measured by the nephelometric reading. The solution was still clear to ordinary observation, and was clinically just as safe and satisfactory for injection as it had been before the heating. This heat treatment was employed, not only in order to insure the sterility of albumin in the final container after it had previously been sterilized by a suitable filter, but also because it effected the destruction of viruses, such as that of infectious jaundice. Thus, it was possible to eliminate the mercurial preservative formerly required in albumin preparations intended for clinical use, and at the same time to obtain added security against the possibility of virus infections resulting from albumin injection. From their studies, Scatchard, Strong, Hughes, Ashworth, and Sparrow concluded that "a mixture of 0.02 molal acetyltryptophanate and 0.02 molal caprylate gives a greater stability at 63°C. than does 0.02 molal acetyltryptophanate at 60°C. The sodium ion concentration is the same, and the tryptophan is sufficient to make the albumin complete for growth in rats. It should be practicable to heat this solution 10 hours at 64°C."

\* The use of the sodium salt of acetylphenylalanine was suggested by Dr. H. T. Clarke at a meeting of the Protein Committee of the National Research Council.

Further evidence of the remarkable capacity of serum albumin to enter into combination with many molecules and ions has continued to accumulate from other sources. Detailed study was made by Rawson (175) of the dye T-1824, which contains several aromatic sulfonic acid groups, and had been used by Gregersen and Gibson (83) for the measurement of blood volume. Electrophoretic studies showed that the dye migrated entirely with the albumin component, up to a concentration of 8-12 moles of dye per mole of albumin. At high concentrations some of the dye migrated with the  $\beta$ -globulin. In the presence of a high concentration of the dye, the mobility of the albumin at pH 7.45 was shifted from approximately  $5 \times 10^{-5}$  to  $7 \times 10^{-5}$ . In the ultracentrifuge, T-1824 in albumin solution moved with the albumin boundary, and the solution was colorless in the region beyond the sedimenting boundary where there was no protein.

Certain other dyes—niagara sky blue 6B, trypan blue, and niagara sky blue—were also bound preferentially by the albumin fraction of plasma, as shown by Rawson's studies; but the binding of the other dyes was much weaker than that of T-1824.

Undoubtedly related to these phenomena is Kendall's (115) finding that crystalline human serum albumin, prepared by ammonium sulfate fractionation, contains about 2% of firmly bound fatty acid. Hughes (40) also found fatty acid, although in smaller amounts, in albumin crystallized from ethanol. His observations on the remarkable effect of decanol, and other higher alcohols, in promoting crystallization of albumin have already been discussed in Part VI.

Another effect of albumin was revealed by the studies of Dubos (55), who showed that serum albumin, either human or bovine, when added to a suitable medium, will permit ready growth of tubercle bacilli, although little or no growth takes place in the absence of albumin. The effect of protein here was traced to its action in removing certain growth inhibitors which belonged to the class of unsaturated fatty acids such as oleic acid. A large number of proteins and other substances were tried, and serum albumin was found to display the highest specific capacity to bind oleic acid. Heat denaturation of the albumin was found to abolish this specific property. The binding capacity was approximately 9 molecules of oleate per molecule of albumin (Davis and Dubos, 47b, Davis, 47a).

The studies of Scatchard, Batchelder and Brown (183) indicate that albumin combines with chloride ions—approximately six per albumin molecule—even when the protein carries a negative net charge.

It has been found by Fieser and Heymans (unpublished) that a large

group of quinones and related substances can be dissolved to form stable aqueous solutions in the presence of albumin, although they are quite insoluble in pure water or salt solution. The mechanism involved here appears to be clearly similar to those of the other components described above, and the binding between serum albumin and these quinones is now being investigated by H. A. Saroff in this laboratory.

A quantitative study of the combination of bovine serum albumin with certain aromatic anions containing sulfonate groups has recently been undertaken by Klotz, Walker and Pivan (118). The ions chosen for study were methyl orange, containing one sulfonate group, and azosulfathiazole, containing two such groups. The solutions were equilibrated across cellophane membranes with the albumin in the inner solution. From the known amount of total methyl orange or azosulfathiazole, and the measured amount in the outer fluid at equilibrium, the amount inside the membrane was calculated, and the amount bound by the protein could be immediately estimated from the excess concentration inside. The results appeared to indicate limiting capacity of about 22 groups of the added ion per mole of albumin at pH 5.7. The form of the combination curves, as a function of concentration of added ion, appeared to indicate a statistical distribution of the binding groups on the surface of the protein, and was compatible with the view that the intrinsic affinity of all these protein groups for the added substance was approximately the same. Electrostatic interactions, particularly in the case of azosulfathiazole, played a definite part in determining the form of the curves when a number of groups on the protein were already occupied.

This combining power has also been demonstrated by observations on the absorption spectra of these same dyes, and also of Orange I and Orange II, in the presence of anionic bovine serum albumin (Klotz, 117). Albumin, at concentrations up to 0.2% produced progressive changes in the position and height of the absorption maximum of the dye (at about  $10^{-5}M$ ). Further increases of albumin concentration produced no further change in spectrum; presumably dye binding was complete at 0.2% albumin. No effect on the spectrum of the dye was produced by gelatin or bovine  $\gamma$ -globulin. Addition of various aliphatic and aromatic carboxylate and sulfonate anions indicated that they competed with the dye anions for combination with albumin. The affinity constants for binding of similar carboxylate and sulfonate anions were much alike, but the presence of additional aromatic rings in the anion greatly increased the affinity constant, presumably through enhancement of van der Waals forces.

These studies are obviously forerunners of a vastly increased number

of quantitative studies of the interactions between the proteins and smaller molecules or ions. Systematic modification of the structure of the protein by reagents having well-defined and specific action on protein groups\* and the determination of the effects of such modifications on the interactions should lead to a far deeper insight into the mechanism responsible for these effects, and provide clues to the nature of the specific differences between different proteins.

(\* See review by Herriott elsewhere in this volume.)

Several further reviews and discussions dealing with this general field are now in preparation: see D. J. Mulford, *Annual Review of Physiology*, 9 (1947); E. Brand and J. T. Edsall, *Annual Review of Biochemistry*, 16 (1947). A comprehensive review of experimental procedures involved in protein fractionation is to be given by L. E. Strong in the forthcoming "Encyclopedia of Chemical Technology"; Fig. 9 of the present review is taken from this article, by the courtesy of Dr. Strong. A "History of Plasma Fractionation", covering the chemical, clinical and immunological studies carried on in this field during the war and the period just preceding the war, is given by E. J. Cohn in Chapter XXVIII of "Advances in Military Medicine: The History of the Committee on Medical Research." The most comprehensive treatment of the whole field is to be given in the Silliman Lectures of E. J. Cohn, to be published in 1948.

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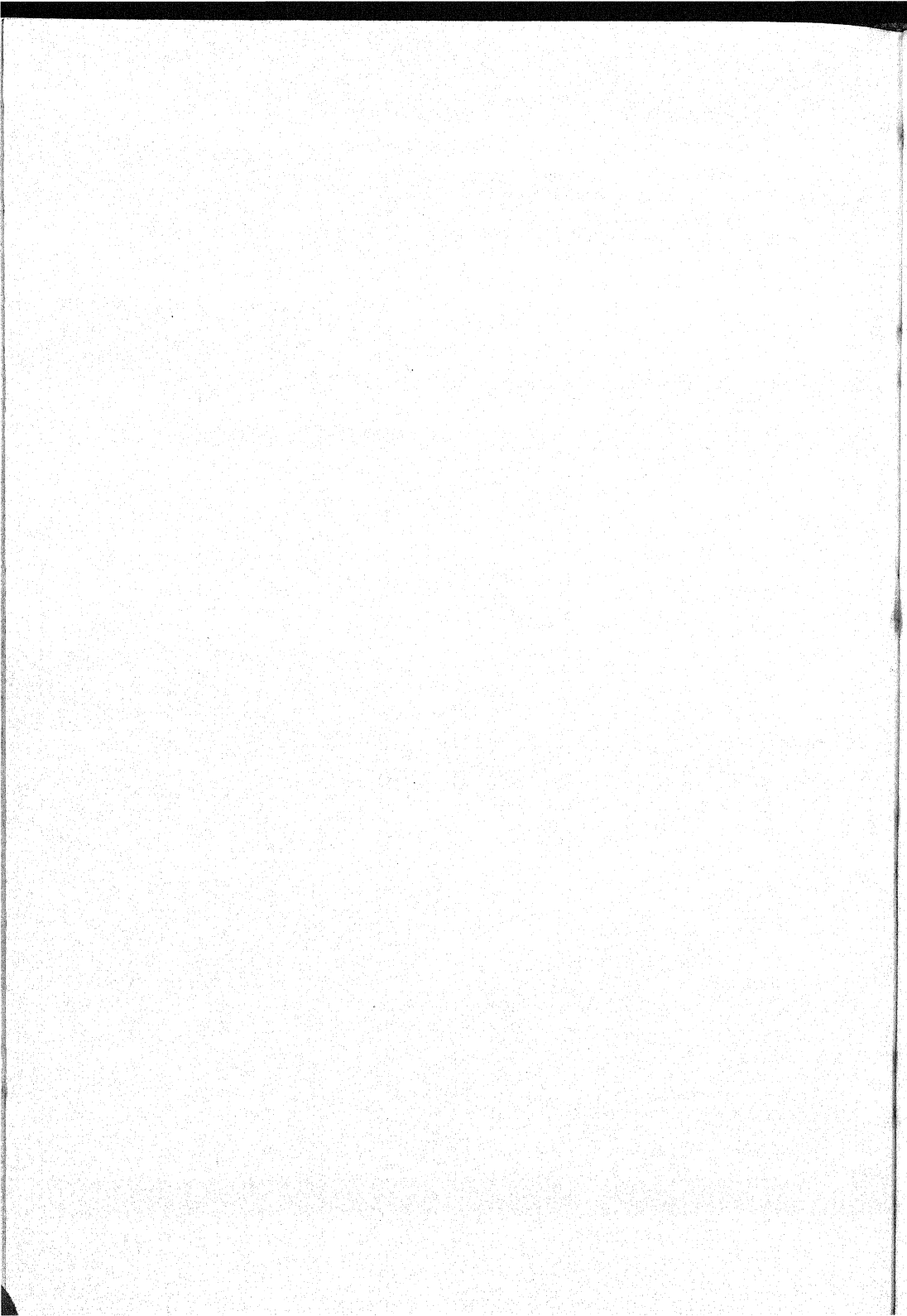


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